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(54) Title: EARLY DIAGNOSIS OF SEPSIS UTILIZING ANTIGEN-ANTIBODY INTERACTIONS AMPLIFIED BY WHOLE BLOOD CHEMILUMINESCENCE

(57) Abstract

A method for determining the extent of an infection in a human or animal patient by detecting the amount of an antigen indicative of such infection, the amount of the antigen is detected in a patient blood derived test sample containing blood cell fractions, the method comprises: i) incubating the test sample with an amount of test antibodies specific to the antigen indicative of infection to form antibody/antigen complexes which are insoluble in the sample, ii) allowing the antibody/antigen complexes to interact with the white blood cell fractions which results in the production of oxidants, iii) introducing to either steps i) or ii) a chemiluminescent compound to the test sample, iv) the oxidants reacting with the chemiluminescent compounds to emit luminescent light from the test sample, v) measuring the amount of emitted light over a predetermined period, and vi) correlating extent of infection by comparison of the measured amount of emitted light of the test sample with measured amount of light emitted by a control sample which is treated the same as the test sample for steps i) to v) except that in step i) control antibodies are used which are of the same class as the test antibodies but are non-specific to the antigens indicative of infection.

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EARLY DIAGNOSIS OF SEPSIS UTILIZING ANTIGEN-ANTIBODY INTERACTIONS AMPLIFIED BY WHOLE BLOOD CHEMILUMINESCENCE

FIELD OF THE INVENTION

This invention relates to a rapid diagnostic screening technology to allow biochemical staging of a patient's infection or septic progression so that an appropriate therapeutic intervention strategy can be identified and initiated.

BACKGROUND OF THE INVENTION

Hospital and particularly intensive care unit patients who have acquired nosocomial infections as a result of peri- or post-operative immunosuppression or secondary to other disease processes, such as, pancreatitis, hypotensive or hypovolemic shock, physical trauma, burn injury, organ transplantation, and develop septic shock syndrome have a mortality which has been quoted to range from 30-70% depending upon other co-incident complications. Until the recent advent of novel new therapeutic strategies these patients have been managed largely by palliative care and administration of antibiotics. The growth of biotechnology has allowed the large scale production of many new target directed biopharmaceuticals which utilize monoclonal antibodies against such initiators of sepsis as gram-negative endotoxin (Centocor's HA-1A or Xoma's Xomen-E5), tumour necrosis factor (various producers including Hoffman LaRoche), Centocor with patents WO90/06514 and WO92/16553), interleukins and various soluble receptor antagonists such as IL-1 RA (Synergen) and sCR_1 (soluble complement receptor 1)-a truncated recombinant complement regulatory molecule.

Despite the development of increasingly potent antimicrobial agents, the incidence of nosocomial infections and, in particular, infections leading to sepsis or septicemia is increasing. The difficulty with many of the promising therapeutic agents is that their window of opportunity and

indications for use have not been adequately delineated largely due to a lack of appropriate rapid diagnostic procedures and partly due to a lack of complete understanding of the pathogenesis of the sepsis syndrome. The cost of these therapeutic agents is significant, being priced at $3,000.00 to $4,000.00 per dose. Thus providing this therapy indiscriminately to patients would add a considerable burden to the health care system without providing a corresponding benefit to patients.

Currently, one of the major problems with many of the therapeutic protocols being tested by the pharmaceutical companies conducting clinical trials in sepsis intervention is their inability to rapidly detect early and evolving sepsis. The results of blood cultures may arrive too late. Other septicaemia tests are also time consuming and may not be sensitive enough for early detection. Centocor Inc.'s immunometric assay for tumour necrosis factor-alpha uses two antibodies, one of which is labelled (WO90/06514). The National Aeronautics and Space Administration detects Pseudomonas bacteria by extraction of Azurin and detection using Azurin-specific antibody (U.S. Patent 7,501,908). The endotoxin assay kit purchased from BioWhittaker (Walkerville, MD., U.S.A.) or Seikagaku Kogyo Ltd. (Tokyo, Japan) is a Limulus Amebocyte Lysate Assay technique which may be used as a comparison for the present invention.

Many investigators versed in the complexities of the septic response believe that treatment is ineffectual for patients who already manifest the classical clinical symptoms of sepsis (ie. hyperdynamic circulation, hypotension, decreased systemic vascular resistance, pyrexia and increased oxygen dependency). The course of the inflammatory process has progressed too far for many of the interventions to benefit the patient since the multiple interacting inflammatory cascades which attempt to eliminate the infectious challenge are in many instances at their nadir and difficult to control pharmacologically. A major clinical and diagnostic challenge is to identify and stage patients, ideally early in the progression of the septic

response, or to identify those patients at high risk of developing fulminant sepsis syndrome. The same therapeutic agents given at the appropriate stage in the septic process may have more significant beneficial effects since it is clear that an optimal window period may exist for the efficacy of any particular therapeutic agent. For example, giving a patient antibodies or receptors directed against gram-negative endotoxins when the patient has no detectable levels of these agents present in the circulation and already has a maximally activated cytokine cascade is a waste of resources and of no benefit to the therapy of the patient. The potential market for these anti-sepsis strategies remains large (about 250,000 cases per year in the USA) and has been limited by the inability to identify and stage patients who could benefit from the appropriate pharmacologic interventions.

In accordance with this invention, a new rapid diagnostic screening technology is provided which determines biochemical staging of a patient's septic progression so that the appropriate therapeutic intervention strategy can be rapidly identified and initiated utilizing either single or multiple agents. Such an approach optimizes the potential cost/benefit ratio by systematically and biochemically identifying patients who would potentially benefit from target directed antisepsis therapy. The lack of such rapid testing strategies combined with the high unit dose cost of many of the new therapies ($3,700.00 US per dose for Centocor HA-1A human IgM antibody) has had a seriously limiting effect on FDA approval. The present invention's analytical approach provides the early diagnostic information necessary for rapid and rational decision making.

Sepsis is defined as the presence of pathogenic microorganisms or their toxins in the blood or other tissues. Septic shock is shock that may develop as a result of a severe infection. Between 60% to 70% of septic shock cases are caused by Gram-negative aerobic bacilli infections. Since both the incidence and mortality accompanying shock are higher in Gram-

negative than Gram-positive infections, most therapy research has been directed to combatting septic shock due to Gram-negative septicemia.

A significant area of therapeutic research is in the development of antibiotic therapy. Although antibiotics do not prevent the toxic effects of endotoxin (and may stimulate the release of endotoxin from bacteria during cell killing), it has been clearly demonstrated that the judicious administration of antibiotics can significantly increase survival, with up to a 50% reduction in the occurrence of shock.

Immunomodulating substances are released as part of the non-specific host immunoinflammatory defense mechanism to gram-negative infections. Some of these substances are mediators of the toxic effects of endotoxin and researchers have developed monoclonal antibodies, receptor antagonists and other agents directed to them. Once again, successful therapy with these immunologic agents depends on their timely administration.

Another sepsis therapeutic treatment involves treatment with monoclonal antibodies directed against lipid A. Two such products are HA-1A (Centoxin) developed by Centocor (Zeigler, E.J., et al. N. Engl. J. Med. 429:324 (1991); Michie, H.R. Proceedings of Brussels Sym. 329 (1992)) and murine IgM MAbE5 (Xomen-E5) developed by Xoma. Given the high price of these treatments, it is essential to predict whether this therapy will even benefit the patient. Further, because Centoxin and Xoma-E5 are only effective in gram-negative septicemia, they will be potentially ineffective in almost 50% of the cases in which they are administered if clinical symptoms are the major criterion for use.

Often the clinical signs of sepsis and sepsis syndrome (the earliest stages) are recognized long before results of blood or other body fluid cultures are available. Without a comprehensive diagnostic test, which is desirably carried out at bedside to indicate sepsis and its level of progression many patients may be treated unnecessarily or with an inappropriate therapeutic regime. In many patients, initiation of monoclonal antibody

therapy against gram-negative endotoxin on the basis of clinical symptoms such as profound hypotension, hyperdynamic circulation, pyrexia, etcetera, may be too late in the disease process. Since many of the proximal cytokine mediators of sepsis are likely to be maximally recruited, effective blockade of the cytokine response or blunting of the initiating effects of endotoxin must be entertained during the early pre-clinical phase. Identification of this early initiating phase of sepsis may be achieved with the present invention's rapid and sensitive assay for both gram-negative and gram-positive endotoxins. If the test is not rapid, the time span of several hours may be sufficient to drive the progression of early profound endotoxin challenge to irreversible sepsis. For this reason our diagnostic strategy is beneficial in providing rapid and if necessary, repetitive feedback regarding septic progression. The present invention provides the comprehensive diagnostic test needed to determine the proper therapy for the patient with sepsis, by quantitatively measuring antigen and mediator levels in the blood. The present invention is equally effective for measuring any antigen present in the blood. Thus the present invention can measure the amount of antigens to provide staging of an infection.

The analytical approach of this invention allows the rapid diagnosis and staging of patients who have this devastating syndrome by harnessing the patients own recognitive systems to help amplify the analytical signal. Our diagnostic approach also constitutes a rapid test for evaluating the efficacy of antibodies directed against specific antigens in whole blood.

This invention takes advantage of the specificity of antigen-antibody interactions and the high sensitivity of chemiluminescent light emission which is triggered in white blood cells through the generation of oxidants and their excitation of a chemilumiphor. A sensitive and generic approach to the detection of sepsis related antigens is thereby provided.

Allen, U.S. Patent 5,108,899 also describes the use of chemiluminescence to detect oxidant production, however, Allen

accomplishes this aspect in a different way for a somewhat different purpose as will be described in respect of the detailed discussion of the inventive preferred embodiments.

SUMMARY OF THE INVENTION

According to an aspect of the invention, a method for determining the extent of an infection in a human or animal patient by detecting the amount of an antigen indicative of such infection, the amount of the antigen is detected in a patient blood derived test sample containing blood cell fractions, the method comprises:

i) incubating the test sample with an amount of test antibodies specific to the antigen indicative of infection to form antibody/antigen complexes which are insoluble in the sample,

ii) allowing the antibody/antigen complexes to interact with the white blood cell fractions which results in the production of oxidants,

iii) introducing to either steps i) or ii) a chemiluminescent compound to the test sample,

iv) the oxidants reacting with the chemiluminescent compounds to emit luminescent light from the test sample,

v) measuring the amount of emitted light over a predetermined period, and

vi) correlating extent of infection by comparison of the measured amount of emitted light of the test sample with measured amount of light emitted by a control sample which is treated the same as the test sample for steps i) to v) except that in step i) control antibodies are used which are of the same class as the test antibodies but are non-specific to the antigens indicative of infection.

According to another aspect of the invention, a method for determining the extent of sepsis in a human or animal patient by detecting the amount of sepsis associated markers in a patient blood derived test sample containing white blood cell fractions, the method comprises:

i) incubating the test sample with an amount of test antibodies specific to a selected sepsis associated marker to form antibody/marker complexes which are insoluble in the sample,

ii) allowing the antibody/marker complexes to interact with the white blood cell fractions which results in the production of oxidants,

iii) introducing to either steps i) or ii) a chemiluminescent compound to the test sample,

iv) the oxidants reacting with the chemiluminescent compounds to emit luminescent light from the test sample,

v) measuring the amount of emitted light over a predetermined period, and

vi) correlating extent of infection by comparison of the measured amount of emitted light of the test sample with measured amount of light emitted by a control sample which is treated the same as the test sample for steps i) to v) except that in step i) control antibodies are used which are of the same class as the test antibodies but are non-specific to the sepsis associated markers.

Infectus or septic indicators include antigens of gram-negative bacteria, gram-positive bacteria, virus, fungus or inflammatory mediators such as tumour necrosis factor, interleukin 1, 6 or 8 and interferons or transforming growth factor β.

In accordance with another aspect of the invention, the test or control sample may be whole blood or white blood cell fractions including neutrophils, lymphocytes and/or monocytes. The addition of zymosan or latex beads and particularly opsonized zymosan or opsonized latex beads enhance the method. The chemiluminescent compound may be luminol, lucigenin or pholasin. The generation of a differential chemiluminescence response between the test sample using specific antibody and the control using a non-specific antibody of the same isotype is not dependent upon the

presence of zymosan or latex but is is dependent upon the presence of plasma.

In accordance with another aspect of the invention, monoclonal antibody of the IgM class directed against the lipid A portion of the gram-negative endotoxin is incubated with a patient's blood. A solution of luminol and then opsonized zymosan is added and chemiluminescence is measured for at least 10 to 20 minutes. This is compared to the chemiluminescence of the patient's blood with IgM antibody non-specific to antigens or mediators indicative of infection.

In accordance with another aspect of the invention, a monoclonal antibody of the IgG class directed against Hepatitis A virus is incubated with a patient's blood. A solution of luminol and then opsonized zymosan is added and chemiluminescence is measured for at least 10 to 20 minutes. This is compared to the chemiluminescence of the patient's blood with IgG antibody non-specific to antigens or mediators indicative of infection.

In accordance with another aspect of the invention, a diagnostic kit for use in determining the extent of infection in a patient by detecting the presence of antigen indicative of infection or mediators in response to infection, in a patient's blood derived test sample containing white blood cell fractions comprises:

i) a first container of IgM or IgG antibody specific to antigen or mediators indicative of infection,

ii) a second container of chemiluminescent compound,

iii) a third container of zymosan or latex beads.

The chemiluminescent compound may be selected from the group of compounds consisting of luminol, lucigen and pholasin. The antibody may be IgM antibody against gram-negative endotoxin lipid A or IgG antibody against Hepatitis A. The zymosan may be opsonized zymosan and the latex may be opsonized latex.

According to a further aspect of the invention, a method for detecting the amount of an antigen in a human or animal patient's body fluid test sample in the presence of white blood cells and all complement proteins, the method comprises:

i) incubating the test sample with an amount of test antibodies specific to the antigen to form antibody antigen complexes which are insoluble in the sample,

ii) allowing the antibody/antigen complexes to interact with the white blood cells and the complement which results in the production of oxidants,

iii) introducing to either steps i) or ii) a chemiluminescent compound to the test sample,

iv) the oxidants reacting with the chemiluminescent compounds to emit luminescent light from the test sample,

v) measuring the amount of emitted light over a predetermined period, and

vi) correlating the amount of the antigen by comparison of the measured amount of emitted light of the test sample with measured amount of light emitted by a control sample which is treated the same as the test sample for steps i) to v) except that in step i) control antibodies are used which are of the same class as the test antibodies but are non-specific to the antigen.

BRIEF DESCRIPTION OF THE DRAWINGS

Preferred embodiments of the invention are demonstrated with respect to the drawings wherein:

Figure 1 is the chemiluminescent response of whole blood with monoclonal antibody and with 100 pg/ml endotoxin and without endotoxin.

Figure 2A is the chemiluminescent response with 5 minute pre-incubation with monoclonal antibody with and without 10 pg/ml endotoxins.

Figure 2B is the same as Figure 2A except that the incubation period with antibody is 60 minutes.

Figure 3A is the chemiluminescent response using blood from a patient with severe sepsis syndrome who died 6 hours after the sample was taken, as compared to a control antibody of the same class, isotype and concentration but directed against irrelevant epitopes.

Figure 3B uses blood from a healthy ambulatory volunteer.

Figure 3C uses blood from a patient with chronic sepsis.

Figure 3D uses blood from a patient with severe sepsis syndrome which contributed to his death 3 days after the sample was taken. This patient had no evidence of gram-negative endotoxemia or bacteremia.

Figure 3E uses blood from a patient being weaned from respiratory support and seriously cachectic, but with no clinical evidence of any septic foci.

Figure 4 is the chemiluminescent response using blood from a patient with a leaky duodenal ulcer.

Figure 5 is the whole blood chemiluminescence response using varying concentrations of endotoxin with varying concentrations of antibody against the endotoxin.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The invention is a sensitive, specific and rapid detection method for antigens indicative of infection in whole blood or other body fluids containing white blood cell fractions and all related complement proteins based upon the specificity of antigen-antibody interactions and the high sensitivity of chemiluminescent light emission. The invention provides early diagnostic information for determining the amount of antigens indicative of the extent of sepsis and the amount of any mediators which provide information to indicate the extent or stage of sepsis. Results are obtained in minutes which is a great advantage over the previous time consuming methods of blood culturing for determining sepsis.

The presence of bacteria, viruses or fungi or their related antigenic components in blood is indicative of an infection. In addition the immune system's reaction to the presence of these foreign antigens by the production of pro-inflammatory mediators such as interleukin-1, tumour necrosis factor and interleukin-6 is also indicative of an infection. The presence and amount of antigens and mediators of sepsis indicates the level or stage of sepsis. For instance, at an early stage of gram-negative sepsis, antibody against gram-negative endotoxin (LPS) in the test of the present invention will detect the presence of LPS at a concentration as low as 5 pg/ml of whole blood. At the next stage, sepsis has progressed and a mediator of sepsis, tumour necrosis factor (TNF-α), can be detected and measured using antibody against TNF-α. At stage 3, TNF-α may be present in smaller amounts since it is transitory and another transitory mediator interleukin-1 (IL-1) may appear. As sepsis progresses further, LPS levels may decrease and TNF-α be absent, but IL-1 may increase and interleukin-6 (IL-6) may appear. Finally, in a more prolonged case of sepsis, LPS may be present and IL-1 may be at low levels but IL-6 may be at very high levels. Thus, the rapid measurement of antigens and mediators can provide a measure of the patient's severity and duration of sepsis and ensure that the correct treatment is administered. The patient's own antibodies if produced to an antigen indicative of sepsis will not appear until 8 to 14 days after exposure to the antigen and thereby do not affect the present invention's antigen detection system.

The present invention measures the amount of antigen and mediators in the patient's blood and provides this information rapidly to determine the patient's level of infection. This rapid test and the staging it provides for a septic patient is critical for the administration of appropriate treatment. If an antigen indicative of sepsis is present in a patient's blood, an antibody specific for the antigen indicative of sepsis can form an antigen/antibody complex at the right concentration of antibody. This is especially effective

with monoclonal antibody. This antigen/antibody complex activates complement which in turn causes neutrophils and other white blood cells to produce oxidants. The white blood cells can be stimulated with opsonized zymosan resulting in increased production of oxidants. The oxidants cause an added chemiluminogenic compound, such as, luminol to release light energy. The amount of light emitted over time from a single test can be measured by a luminometer device to indicate the amount of antigen and hence the presence and extent of sepsis. The luminometer may be a Berthold model 481 luminometer which is available from Berthold Instruments Inc. in Austria. The addition of opsonized zymosan enhances the chemiluminescent response. A comparison control sample of the patient's blood is prepared by combining the blood with antibody of the same class non-specific for antigens indicative of sepsis.

The use of chemiluminescence to detect oxidant production is also described in the Robert C. Allen U.S. Patent 5,108,899. The Allen patent, however, measures inflammation of a patient by comparing the extent of opsonin receptor expression on phagocytes with the maximum inducible opsonin receptor expression. The theory is that the less opsonin receptor expression may be induced, the greater the inflammation. The Allen patent uses agents which bind to phagocytes and stimulate opsonin receptor expression to give a maximum amount of chemiluminescence with zymosan.

In contrast, the present invention adds an antibody specific for an antigen or mediator of sepsis or infection. If an antigen or mediator is present then antibody/antigen or antibody/mediator complex is formed. The formation of antigen/antibody complexes in blood results in the activation of the classical complement pathway in the case of IgM complexes via C1q with the formation of complement anaphylatoxins and lytic products of C3, C4 and C5. A heat labile element produced during this process is responsible for the activation of white cell oxidant production (see Example XII) which results in the oxidation of luminol with subsequent emission of

light. IgG immune complexes may modulate white cell oxidant production and chemiluminescence via a different mechanism which could involve binding to white cell Fcγ receptors and subsequent formation of membrane attack complex followed by cell lysis. Leukocytes do not have Fc receptors for IgM immune complexes. In the present invention, a patient's blood is tested for the level of antigens and mediators which quantities indicate the stage of sepsis. Example X demonstrates that activation of white blood cells by immune complex formation is by a mechanism other than opsonin receptor formation. The Allen patent only indicates how much further opsonin receptor expression may be induced, but unlike the present invention, it does not indicate which antigens or mediators are present and at what levels nor does it provide the stage of sepsis.

Although zymosan or latex beads are not a required in addition to the test procedure, the measure of immune complexes in the test sample as compared to the control is enhanced by the addition. Zymosan and latex beads enhance the chemiluminescent response by stimulating concerted white cell oxidant production and phagocytosis. The addition of zymosan or latex acts as an amplification process to increase oxidant production but is not obligatory for the recognition of immune complex formation. The enhanced production of white cell oxidants stimulated by IgM immune complexes is not dependent upon increased opsonin receptor expression since the addition of a maximal stimulatory dose of C5a (which upregulates maximal opsonin receptor expression) to the blood during immune complex formation does not significantly affect the ratio of control to specific antibody dependent chemiluminescence when expressed as a light integral or peak count per minute either in the presence or absence of opsonized zymosan (see Example X).

According to an aspect of the invention, a sample of the patient's whole blood may be tested for the indication of sepsis. The sample of whole blood is mixed preferably with a monoclonal antibody, such as,

Xomen-E5, produced by Xoma in Palo Alto, California, a murine monoclonal IgM pentamer directed against the lipid A component of gram-negative endotoxin. Lipid A is the toxic inner core portion of the lipopolysaccharide (LPS) which is highly conserved and thereby is present on all gram-negative bacteria. The blood, which may contain endotoxin, is incubated with the monoclonal antibody. In parallel, a non-specific control murine antibody of the same isotype is incubated with a sample of the blood for comparison. To the blood-antibody mixture is added luminol solution and then complement activated zymosan or complement opsonized latex beads.

All analyses are preferably carried out in triplicate and the complete assay mixture in polystyrene or glass cuvettes is placed in a thermostated (37°C) chemiluminometer for repeated multiple readings of light emission intensity. In order to provide a semi-quantitative estimate of the amount of endotoxin in the blood sample the analysis, is conducted, in accordance with one aspect of the invention, using 3 different dilutions of specific and control antibody each of which differ from the next highest concentration by one order of magnitude (ie 1:10, 1:100, 1:1000 dilution). The presence of antigen of interest, in this case, gram-negative endotoxin is confirmed by a statistically significant increase in integrated light intensity or reaction slope during the first 10 to 20 min. of reaction. The three different concentrations of antibody are used to discriminate and semi-quantitate the amount of endotoxin which is present. The principle of the triple concentration approach is based on the observation that maximal stimulation of white cell chemiluminescence in whole blood occurs when antigen-antibody complementarity is optimal for the formation of macromolecular crosslinked complexes or aggregates. In the presence of high concentrations of antigen, a high antibody concentration is required to yield such optimal complementarity. Similarly, at intermediate and low concentrations of antigen less concentrated antibody is required for optimal complementarity

and macromolecular aggregate formation. This basic principle has been used for years in Ouchterlony diffusion plates and radial diffusion plates for immunometric quantitation of precipitin reactions. The whole blood chemiluminescent approach provides a semi-quantitative determination of the antigen concentration in question as high, intermediate or low with analogous concentration ranges (ie. >100 pg/ml, 20-100 pg/ml, <20 pg/ml). Thus the maximal stimulation of chemiluminescence will occur for the 1:10 antibody dilution when the antigen level is at >100 pg/ml; for the 1:100 antibody dilution when the antigen level is at 20-100 pg/ml; and for the 1:1000 antibody dilution when the antigen level is at <20 pg/ml.

A whole blood sample may be used directly in this invention which simplifies the procedure, allowing it to be conducted bedside and provides the desired level of chemiluminescence. However, diluted whole blood or white blood cell fractions may also be used in this invention. Besides being more time consuming, fractionating procedures may result in a loss of subpopulations of neutrophils (Ristola, M. and Repo H., APMIS 503:97 (1989)). Using whole blood is much faster, easier and provides the chemiluminescent response above background noise and capture of light energy. An added potential advantage of the utilization of whole blood rather than plasma or serum as the sample and partial reagent, is that it allows the detection of antigens which are cell associated or bound but still expose accessible epitopes. The use of antibodies against such epitopes may allow antibody binding with the receptor or binding sites on cells permitting a positive analytical signal which would not otherwise be detectable in a plasma or serum sample alone.

The antibody against an antigen indicative of sepsis or against a mediator can be of the IgM class. Unlike IgG, IgM does not bind to the Fc receptors on the white blood cells. IgM-antigen complexes trigger a reaction sequence which results in the stimulation of white blood cell oxidant production via a heat labile plasma component. Thus white blood cell

activation is dependent upon a classical complement pathway activation which is triggered by the formation of antigen-antibody complexes.

The antibody against an antigen indicative of sepsis or against a mediator can also be of the IgG class. IgG binds to the Fc receptors on the white blood cells and causes cytolysis. There is a decrease in the level of white blood cell activation from the normal level since white blood cells are lysed. This then also provides a semiquantitative measure of the level of antigen present and shows an especially dramatic drop in white blood cell activation with high levels of antigen or mediator.

The chemiluminescent response is measured for at least 10 to 20 minutes to determine the extent of sepsis in comparison to a control. Chemiluminescence may be measured for an hour or longer, but over time the neutrophils appear to be progressively deactivated or their chemiluminescent response is blunted or an inhibitory luminol metabolite is formed.

Zymosan is a fungal polysaccharide which is optionally used to stimulate further, phagocytic cells. It is well known that phagocytic cells elicit a maximal phagocytic response when they can ingest particulate matter particularly via receptor mediated mechanisms. Although untreated zymosan can stimulate the phagocytic response, a much more exaggerated response can be achieved by opsonizing the zymosan first. The process of opsonization for this invention involves the binding of IgG's and complement factors iC3b and C3b to the zymosan. This process allows the opsonized zymosan to bind to three different classes of receptors on the phagocytic cells. IgG's bind to specific Fc receptors, C3b coated particles bind to CR_1 receptors and iC3b coated particles bind to the CR_3 receptor. Non-opsonized zymosan binds only to a subsite of the CD11b/CD18 integrin receptor which is different from the CR_3 binding site. Due to its multiplicity of binding sites opsonized zymosan triggers a much more vigorous respiratory response in phagocytic cells than a comparable concentration of

non-opsonized zymosan. In this invention opsonized zymosan is used to maximally elicit the oxidative response of the white blood cells. Opsonized zymosan white blood cell activation is dependent upon the concentration of CR_1 and CR_3 receptors on the surface of the phagocytes. The best results used complement opsonized zymosan (minimal IgG), however any type of opsonized zymosan may be used, for example, IgG opsonized or complement and immunoglobulin opsonized. It was also shown that complement opsonized latex beads could be used to maximally elicit the oxidative response of the white blood cells.

The preferred aspect of this invention uses a small volume of undiluted whole blood (20 μl or 50 μl) heparinized (< 2 U/ml) or EDTA anticogulated and kept at room temperature. The blood sample is incubated with an equal volume of antibody (0.2, 0.02 and 0.002 mg/ml) at 37°C for five to ten minutes. After incubation, 200 μl of 40 μM luminol solution is added followed by 50 μl of complement opsonized zymosan. 2.5 - 3.0 x 10^9 particles/ml. The sample is read in thermostated (37°C) luminometer.

In another aspect of this invention whole blood is first combined with luminol solution and then antibody is added and the mixture incubated at 37°C for five to ten minutes. Zymosan may then be added.

The examples describe alternatives to these aspects, such as varying the order in which to add the reagents, varying blood dilutions, and omitting zymosan. However, the above aspects provide a better evaluation of the presence and degree of sepsis. Modifications of these protocols will still be within the scope of the invention. The whole blood sample may instead be sub-fraction of white blood cells, such as neutrophils or lymphocytes or monocytes. Another chemiluminescent compound besides luminol may be used, such as, lucigenin or pholasin.

The phenomenon of chemiluminescence resulting from the production of neutrophil oxidants is described by Allen, R.C. Methods in Enzymology 133:449 (1986) using the acyl azide dye luminol as a light emitting agent.

This technique permitted the sensitive measurement of neutrophil respiratory burst activation using small numbers of polymorphs or later even white cells in whole blood. The exact mechanism of light emission from the dye luminol has not been elucidated to date but is thought to involve a chlorinated intermediate produced upon reaction of the dye with hypochlorite at neutral pH. An excited state proceeding through an "activated aldehyde" intermediate is thought to relax to ground state by emission of light at about 450 nm. The quantum yield of this process is of the order of 1%, that is, only 1% of the energy of reaction is emitted as light. The major oxidant produced by white cells, which is thought in accordance with this invention to excite luminol, has been shown to be HOCl although a superoxide anion which is also an oxidant produced by white cells can also excite the dye predominantly at alkaline pH. The primacy of HOCl as the oxidant species which triggers luminol dependent chemiluminescence has been confirmed in whole blood studies using sodium azide as an inhibitor of myeloperoxidase (Allen, R.C. Methods of Enzymology 133:449 (1986). Under these conditions greater than 95% of the chemiluminescent light emission is blocked. Other chemiluminescent dyes which produce light as a result of neutrophil oxidant production have also been identified including lucigenin and pholasin.

Experiments conducted with azide as an inhibitor of myeloperoxidase (Pauksens, K. et al. Scand. J. Infect. Dis. 21:277 (1989)) as well as other studies using purified human neutrophils suggest that polymorphs are the major contributors of oxidants and myeloperoxidase enzyme in whole blood chemiluminescence studies which employ luminol as the primary lumiphor. The major stimulation of whole blood respiratory burst activity occurs when the white cells, particularly the polymorphs are presented with a phagocytizable particle (1-2.5 micron in diameter) which is opsonized with complement or immunoglobulin products. Phagocyte contact with

complement or immunoglobulin opsonized foreign bodies such as microbes results in a programmed sequence of events. This includes:

a) phagocyte recognition of the opsonized material by the opsonin-specific receptors CR_1, CR_3 (CD11b/CD18) and Fc receptors,

b) engulfment or phagocytosis of the foreign body if it is of appropriate dimensions,

c) activation of microbial redox metabolism.

The respiratory burst activity provides reducing equivalents for univalent reduction of oxygen via the hexose monophosphate shunt in the form of NADPH which is used as an electron source for the membrane associated oxidase complex which generates superoxide anion O_2^- into the phagolysosome or the extracellular space. This primary oxidant production in concert with the degranulation of azurophil granules results in the formation of more powerful oxidants such as HOCl which act in concert with neutrophil proteases to kill bacteria and inactivate viral particles.

Allen has proposed in his aforementioned U.S. patent, the use of whole blood chemiluminescence elicited by complement or immunoglobulin opsonized zymosan as a technique to evaluate the degree of white cell and particularly polymorph activation in patients who have activated immune responses. He has devised a kinetic method for measuring neutrophil opsonin receptor expression which is based upon stimulation of whole blood with saturating concentrations of opsonized zymosan. The zymosan maximally stimulates the expressed CR_1 and CR_3 receptors resulting in oxidative burst activity and degranulation which in the presence of luminol transduces the oxidant burst into light emission which can be integrated and quantitated. In order to maximally induce all available opsonin receptor expression (both ambient and latent) a separate analysis is conducted with a maximum stimulatory dose of C5a. The ratio of ambient to maximally

induced chemiluminescence in the presence of complement anaphylatoxin C5a provides an index of inflammatory status which is independent of white cell or polymorph concentration. It has been shown that the instantaneous level of opsonin receptor concentration in a patients white cell cohort is dependent upon the concentration of pro-inflammatory mediators (PAF, LTB_4, C5a, etc) which are in contact with the white cells at any one time. The measurement of opsonin receptor reserve, therefore, provides a rapid functional assessment of inflammatory status which can be used as a prognostic manner. The Allen method does not measure the presence of antigens indicative of sepsis or mediators in the blood. The present invention does measure the presence of such antigens or mediators by the formation of immune complexes using exogenously administered antibody. The Allen method instead measures the degree of phagocyte activation and oxidant production via opsonin receptor expression. The present invention uses the white blood cell excitation to measure the presence of antigens indicative of sepsis and mediators.

The use of chemiluminescence to detect superoxide anion formation triggered by lipoteichoic acid-antibody interaction has only been previously described in the context of purified neutrophils which were pre-coated with lipoteichoic acid and subsequently challenged with antibodies against lipoteichoic acid (Ginsberg, I., et al. Inflammation 12:525 (1988)). Lipoteichoic acid is known to non-specifically adhere to neutrophils via its lipid moiety. The addition of antibodies against lipoteichoic acid triggered a small respiratory burst activity which was shown to be dependent upon prior coating of the neutrophils with lipoteichoic acid and subsequent cross linking or agglutination of neutrophils which was induced by antibody acting as a bridging ligand between lipoteichoic acid on the surface of one neutrophil binding to the Fab portion of the antibody followed by binding of adjacent neutrophils via Fc receptors. In these studies antibodies of the IgG class were used. The authors of this study did not recognize the fact that luminol

detected primarily HOCL production in their purified neutrophil preparations and did not comment on the obvious differences in the kinetics of oxidant production observed in the presence of luminol or cytochrome "c" as detectors of oxidant production.

The present invention's analytical approach is based upon a different mechanism of signal amplification. In one aspect of the invention, the antibodies of the murine monoclonal IgM class are directed against gram-negative endotoxin lipid A (a component of lipopolysaccharide (LPS)). These antibodies do not have complementary Fc receptors on neutrophils or any other white cell subtype (Unkeless, J.C., Boros, P., Fein, M., Inflammation, Basic Principles and Clinical Correlates, 2nd Ed., 497-510; Gallin, J.I., Goldstein, R., Synderman, R., Editors Raven Press, N.Y. (1992). The detection of fluid phase antigen-antibody complexes is based on their ability to fix complement and stimulate white cell oxidant production compared to a control sample containing an irrelevant isotype-matched antibody. The subsequent stimulation of the neutrophils with a non-rate limiting dose of zymosan allows an enhanced signal to be generated by maximal stimulation of phagocytosis. In the absence of antigen no such enhanced response is elicited. Complement opsonized zymosan is added to maximize the phagocytic response of neutrophils.

For the purpose of analyzing patient samples, which may or may not contain antigen, a control comparison sample is co-analyzed with an identical subclass and concentration of antibody which is directed against an irrelevant epitope not found in human blood. By comparing the chemiluminescent response in the control tube with irrelevant antibody to the tube containing specific antibody against the antigen of interest, it is possible to obtain a semi-quantitative estimate of the concentration of antigen after an assay period of 20 minutes or less. The presence of antigen is confirmed by an enhanced chemiluminescence response during the early assay period in the tubes containing specific antibody. One of the examples confirms that

E. coli bacteria added to the blood test sample had no effect on neutrophil chemiluminescence in the absence of antibody against LPS. Endotoxin could eventually cause neutrophil activation however, very slowly compared to the rate of activation of the antigen/antibody complexes.

In another aspect of this invention, antibodies of the murine monoclonal IgG class are directed against Hepatitis A virus. These antibodies do have complementary Fc receptors on white blood cells. The binding of the antigen/antibody complex on the Fc receptors causes the white blood cells to lyse. The lysis of white blood cells at the right concentration of antibody reduces the baseline level of oxidative metabolism and is an indicator of the presence of Hepatitis A virus. Thus any IgM/antigen complexes will activate complement and cause increased oxidant production of white blood cells, whereas IgG/antigen complexes may bind to Fc receptors on white blood cells and cause them to lyse, thus reducing oxidant production from baseline levels.

A unique feature of the present invention is that the patients own finely tuned immune recognitive systems are used to provide the analytical signal. This approach is unique because the patients blood is pre-incubated with either control or target antibody for five to ten minutes prior to the addition of luminol and opsonized zymosan and then the chemiluminescent light emission signal is measured for a period of 10 to 20 minutes. A comparison of calculated slopes or integrated light intensity of the response to control or specific antibody then allows a decision to be made regarding the presence or absence of the antigen. In order to guarantee optimal reaction conditions for antibody reaction in different blood samples three concentrations of antibody are used, each with a matched control. This approach allows rapid and extremely sensitive detection of gram-negative endotoxin at a detection limit which is comparable or lower than many conventional Limulus Amoebocyte Lysate (LAL) assays which require extensive blood sample pre-treatment and are much more labour intensive

and prone to environmental contamination. It also uses whole blood rather than serum or plasma. The results of the present invention's CL (Chemiluminescence) method are compared favourably to the LAL assay method in Example VIII. A preferred embodiment of process steps of this invention is described in Example IX and illustrated by Figure 4.

EXAMPLES

Example I - Verification of Antibody (Xomen-E5) Binding to Gram-Negative Endotoxin as Assessed by Turbidimetric Assay.

Murine monoclonal antibody (Xomen-E5, stock concentration 2 mg/ml supplied in sterile pyrogen free saline) against gram-negative endotoxin was diluted 10 to 10,000 fold in sterile, pyrogen free Hanks Balanced Salt solution (HBSS) at 10 fold increments. Solid polyethylene glycol 6000 was added to the antibody dilutions to give a final concentration of 6% w/v. The Purified gram-negative endotoxin (E. coli serotype 055:B5, grade V, Sigma St. Louis Miss.) was dissolved in HBSS at concentrations of 100, 10, 1 and 0.1 μg/ml (w/v). These two solutions were placed in a Cobas Fara centrifugal chemistry analyzer (Roche Diagnostics, Hoffman La Roche, Miss., Ont.) and equivalent 100 μl volumes were pipetted by the analyzer into cuvettes and optical density measured repeatedly at 340 mn at 30 sec. intervals for a total period of 5 minutes (Table 1).

To confirm that the monoclonal antibody reacts with the endotoxin, a turbidimetric antigen-antibody binding analysis was conducted. Table 1 summarizes the turbidimetric antigen-antibody interaction of the monoclonal murine IgM antibody directed against the Lipid A epitope of gram-negative endotoxin with purified E. coli gram-negative endotoxin (LPS). As demonstrated in Table 1, it is evident that under the conditions of this assay, at an antibody concentration of 2 μg/ml, a dose response relationship is established against increasing concentrations of LPS (from 1 to 100 μg/ml). This experiment confirms the ability of the antibody to recognize LPS and

bind to it to form insoluble antigen-antibody complexes at appropriate concentrations of each reactant which yield optimum complementarity for the precipitin reaction. These results provide, therefore, direct evidence that the antibody chosen for subsequent assay development is capable of recognizing the antigen of interest.

Example II - Effect of Antibody Concentration on the In Vitro Chemiluminescent Response of Whole Blood Containing Gram-negative Endotoxin

Whole blood was collected from the antecubital vein of a healthy volunteer in an equal volume of HBSS containing sodium heparin at a concentration of 2 Units per ml. E. coli endotoxin was added to the whole blood at a final concentration of 1 ng/ml. The following solutions were then added to chemiluminescence cuvettes:

200 μl HBSS containing a saturating conc. of luminol (approx. 40 μM), (This solution will be called luminol solution),

100 μl of Heparinized Whole Blood (Diluted 10 fold with HBSS),

30 μl of antibody solution (final concentration in the total reaction mixture ranging from 100 μg/ml to 0.01 μg/ml in 10 fold dilutions).

All reaction mixtures were assayed in triplicate. This reaction mixture was temperature equilibrated for 5 minutes at 37°C in the reaction chamber of a Berthold model 481 luminometer and 50 μl of complement opsonized zymosan (2.5 - 3.0 x 10^9 particles/ml pre-warmed to 37°C was added to each cuvette. The samples were then read repetitively for a period of 60 minutes in the luminometer at a constant temperature of 37°C. Assays were organized so that every 6 cuvettes contained the same concentration of antibody but only the last three cuvettes of the six contained 1000 pg/ml of endotoxin.

The data in Table 2 illustrates the chemiluminescent response to endotoxin at varying concentrations of anti-LPS antibody. Each group of six

cuvettes contains the same concentration of antibody ranging from 100 μg/ml to 0.01 μg/ml in 10 fold increments. The last three tubes in each group contain blood which was supplemented with endotoxin to a concentration of 1 ng/ml. The integrals of the cumulative light intensity are used to determine whether a significant difference exists between triplicate tubes with and without endotoxin at any particular antibody concentration. As demonstrated in Table 2, the highest concentration of antibody yielded a suppression in chemiluminescence when endotoxin was present and the only other antibody concentration which yielded a differential response was the lowest concentration of 0.01 μg/ml. In this group the presence of gram-negative endotoxin yielded an enhancement of chemiluminescence. For the purposes of assay development, an augmentation of chemiluminescence (CL) is preferable since it is more likely to span a broader range of responses to varying concentrations of endotoxin and the lower requirement for antibody would decrease the cost of the assay. The suppression of the CL response at high concentrations of antibody may be due to destruction of neutrophils bound with endotoxin-anti-endotoxin antibody complexes due to complement mediated lysis of the cells or the inhibition of zymosan activation due to blockade of opsonin receptors by antigen-antibody-complement complexes.

Example III - Chemiluminescent Response of Whole Blood In Vitro With and Without Gram-negative Endotoxin, and with Monoclonal Antibody Against Endotoxin in the Presence and Absence of Endotoxin

One millilitre of whole blood was mixed with endotoxin to yield a final concentration of 1 ng/ml and a duplicate aliquot was mixed with an equivalent volume of carrier (50 μl HBSS). Chemiluminescence cuvettes were then filled in triplicate with 200 μl of luminol solution and 100 μl of blood with and without endotoxin. This blood was pre-diluted ten fold with heparinized HBSS prior to addition to the chemiluminescence cuvettes. To these cuvettes a solution of opsonized zymosan was added (50 μl) along with

25 μl of HBSS. Chemiluminescence was then measured for 60 min (Table 3A).

In a parallel series of experiments the same tubes were prepared as above except that tubes were filled with 25 μl of HBSS containing monoclonal antibody against gram-negative endotoxin (final concentration of antibody 1.3 μg/ml) (Table 3b).

Example III was designed to examine whether endotoxin alone had any effects on CL in the absence of antibody under the conditions of the assay. At a dose of 1000 pg/ml of whole blood the endotoxin had no effect on the chemiluminescence response. In Table 3A none of the samples contained antibody. As indicated in Table 3b the addition of antibody to a final assay concentration of 1.3 μg/ml had no effect on the shape or magnitude of the CL response in the absence of endotoxin. The presence of endotoxin, however caused an augmentation of the CL response both in terms of increased CL maximum, and increased initial acceleration slope observed during the first 15 minutes of the reaction. These results confirmed the observation that the presence of antigen-antibody complexes could be used to enhance zymosan activated whole blood chemiluminescence and hence detect whether LPS was present in whole blood.

Example IV - Optimization of Chemiluminescent Response to Two Different Endotoxin Concentrations (100 pg/ml and 1000 pg/ml) by Varying the Antibody Concentration

Three 1 ml samples of whole blood anticoagulated with EDTA collected from one donor were mixed with 10 μl of HBSS. One of the 10 μl aliquots of HBSS contained 100 pg of endotoxin and the other 10 μl aliquot contained 1000 pg of endotoxin. Each sample either with or without endotoxin was then diluted ten fold with HBSS containing 2 U/L of sodium heparin. The following assay protocol was then used: 200 μl of luminol solution, 100 μl of 10X diluted blood, 25 μl of monoclonal antibody against

endotoxin and 50 μl of complement opsonized zymosan. The final concentration of monoclonal antibody in the reaction mixture was varied from 0.2 μg/ml to 0.0025 μg/ml in dilution increments of 3 fold. All assays were analyzed in triplicate and the reactions were initiated by the addition of opsonized zymosan to the reaction mixture. The chemiluminescent response was monitored for 50 minutes at 37°C. Chemiluminescent curve integrals were taken from the time of zymosan addition until 5 minutes of the initial acceleration phase of the reaction for comparison of responses. All integrals were compared to the parallel control containing an equivalent concentration of monoclonal antibody but no endotoxin (Table 4).

The possibility that one antibody concentration could span a range of CL response from 0 to 1000 pg/ml of endotoxin was investigated in Example IV. The assay results are summarized in Table 4. After careful inspection of the data obtained over a 60 minute assay period it was observed that the best signal to noise ratio was achieved by considering CL curve integrals over the first five minute acceleration phase of the reaction. This data is tabulated in the "Integral" column of Table 4. The starting antibody dilution in this experiment was 0.2 μg/ml. All subsequent dilutions of antibody were made in three fold steps. It is clear from this data that the maximal response ratio between control cuvettes with no endotoxin and cuvettes containing blood with an endotoxin concentration of 100 pg/ml was achieved at the highest concentration of antibody tested, namely 0.2 μg/ml. The response ratio at this concentration was 2.1. At an LPS concentration of 1000 pg/ml the maximal response ratio was achieved at an antibody concentration of 0.007 μg/ml. At this antibody concentration the response ratio for the 1000 pg/ml standard was 1.7.

These experiments highlight the fact that no one single antibody concentration can give a significant signal to baseline response at both these concentrations of endotoxin. The preferred systematic approach with regard to patient samples is to assay for the presence of endotoxin at several

concentrations of antibody in order to maximize the probability of detection over a wide possible range of endotoxin concentrations which are likely to occur physiologically in patients. These results also highlight the observation that the amount of antibody used in the assay modulated the baseline chemiluminescent response in the absence of endotoxin suggesting that any patient based assay protocol should employ an equivalent concentration of an irrelevant antibody which would give a CL response indistinguishable from the specific antibody in the absence of the antigen of interest.

Figure 1 graphically presents the CL data obtained from the reaction mixtures which gave the largest response ratio for endotoxin at a concentration of 100 pg/ml of whole blood. The plotted data emphasizes the difference in the initial slope of the reactions and in the CL maxima. An adequate differentiation of the signals was clearly evidenced after only 5 minutes of reaction emphasizing the rapid diagnostic potential of the assay. The standard chemiluminomiter measure emitted luminescent light by use of the standard type of electronic photo counter. Periodically as plotted along the X-axis in minutes the light emission is measured based on the photon counts per minute (cpm). The cpm value is then plotted on the Y-axis whereby over time the receptive curves are developed. The statistically significant difference in height of the curves is due to correct ratio of antigen to antibody which forms the insoluble complex. As previously described, the ratio defines the corresponding range for the amount of antigen present in the patient sample.

Example V - Chemiluminescent Response of Whole Blood Containing In Vitro Added LPS (Gram-negative Endotoxin) with Addition of Antibodies to LPS but no Zymosan. (ie Chemiluminescent Response in the Absence of an Opsonin Receptor Agonist)

A) These experiments were conducted using the following reaction mixture: 200 μl luminol solution, 100 μl of whole blood diluted 1OX with Heparinized HBSS containing an original undiluted endotoxin concentration of 0, 10, 50 and 100 pg/ml and 50 μl of monoclonal murine anti-LPS antibody (conc. 0.2 mg/ml final concentration in the assay mixture 28.5 μg/ml). The chemiluminescent reaction was initiated by luminometer controlled injection of the diluted blood into the cuvettes and subsequent reading of chemiluminescence (CL) intensity after a 0.5 second delay (Table 5c).

B) In a separate experiment the CL response of anti-endotoxin and control non-specific murine myeloma IgM to 1 ng/ml of endotoxin in whole blood was evaluated using equivalent concentrations of antibody (28.5 μg/ml) using the same experimental protocol as in part A (Table 5a and b).

It has been well established that human neutrophils do not have Fc receptors which recognize IgM of human or murine origin. The source of the CL signal which is observed in the presence of zymosan could not be due to a direct interaction of antigen-antibody complexes with neutrophil Fc receptors resulting in a cross linking of Fc receptors which is known to stimulate respiratory burst activity. In order to investigate the possible mechanism of neutrophil chemiluminescence stimulation observed with our IgM antibodies of both specific and non-specific epitope recognition, the CL signal generation in the absence of exogenous zymosan activation which maximally recruits available surface expressed opsonin receptors was examined. As illustrated in Table 5, addition of equivalent concentrations of the non-specific control antibody and the anti-LPS antibody in the absence of endotoxin resulted in no activation of the white cell population. Addition of

endotoxin, however, resulted in a biphasic increase in CL response in the samples containing anti-LPS antibody only. The non-specific control antibody has only a small stimulatory effect. The excitation of CL in whole blood as a result of antigen-antibody complexes of the IgM class implies that the augmented CL response is triggered by factors other that Fc receptor occupancy, such as, release of complement products or other proinflammatory molecules generated as a consequence of specific antigen-antibody complexes. Another feature of these results is the difference in magnitude of the CL signal generated by antigen-antibody complexes in the absence of zymosan. The intensity of the CL signal with zymosan is approximately 50-100 times higher than without. For this reason opsonized zymosan is used in this invention to increase the magnitude of the response and improve signal to noise characteristics. As indicated in Table 5, Panel C, there was a dose response relationship between endotoxin concentration and the magnitude of CL integral. The biphasic nature of the CL response implies that the early peak which occurs in the presence of LPS is due to intracellular oxidant production and the later progressive rise is due to extracellular oxidant release which is temporally later in the sequence of cell activation. The general assay protocol may be used without the inclusion of opsonized zymosan to prime and excite a maximal respiratory burst activity.

Example VI - Effect of Pre-Incubation Time with Monoclonal Antibody on Chemiluminescence Response in the Presence of Exogenously Added LPS

In this experiment concentration of blood used in the assay mixture was altered in order to try to increase assay sensitivity. Rather than diluting the blood 1OX with heparinized HBSS prior to assay the blood was diluted only 2 fold with the same buffer. In this experimental series we decreased the luminol volume down to 100 μl and used 100 μl of blood (diluted 2X) which had an original endotoxin concentration of 10 pg/ml or no

exogenously added endotoxin. Fifty microlitres of monoclonal antibody was used in the assay protocol (conc. of antibody ranged from 0.2 mg/ml to 2 ng/ml in the 50 μl aliquot).

A) In one series of experiments the blood (diluted 2X) was pre-incubated with 50 μl of antibody solution for 5 minute at 37°C prior to addition of luminol solution and 50 μl of complement opsonized zymosan to initiate chemiluminescence (Figure 2A, Table 6a). This assay protocol is the preferred embodiment of the invention.

B) In a separate parallel series of experiments the pre-incubation period with antibody was extended to 60 minutes prior to the addition of luminol solution and zymosan. (Figure 2B, Table 6b). In both experimental series A and B the CL response was monitored for 60 minutes after the addition of zymosan

It is an advantage of this invention to diagnose low levels of endotoxin as it is easier to treat early sepsis. The CL response to low doses of endotoxin (10 pg/ml of whole blood) was optimized by increasing the volume fraction of blood in the total reaction mixture and also diluting the whole blood only 1:1 rather than 1:9 as in previous assays. These modifications are designed to push the limits of sensitivity high enough to allow very early detection of endotoxemia in order to attempt to detect gram-negative endotoxin in the blood well before catastrophic activation of the immune response is triggered. As indicated in Table 6a, which summarizes the response to LPS with a short pre-incubation of antibody for 5 minutes, the best response ratio was achieved at an antibody concentration of 200 μg/ml which resulted in an almost 2 fold signal enhancement in the presence of 10 pg/ml of endotoxin. Table 6b summarizes a parallel experiment except that samples were pre-incubated with anti-LPS antibody for 60 minutes prior to the addition of opsonized zymosan. In this case pre-incubation with antibody for 1 hour resulted in a decreased CL response in

the endotoxin containing samples with an antibody concentration of 200 μg/ml. This response suggests that prolonged incubation times may have allowed complement activation to proceed to the formation of the membrane attack complex with lysis of neutrophils. Alternatively the prolonged incubation times may have allowed the opsonized antigen-antibody complexes to bind to CRl and CR_3 receptors on the neutrophils and block the binding of complement opsonized zymosan to these opsonin receptors. Figure 2A exemplifies the maximal stimulatory effect observed with the short pre-incubation period. Figure 2B graphically illustrates the inhibitory effect which occurs with identical antibody concentrations but a long pre-incubation time. The short pre-incubation strategy is preferred since it allows signal amplification rather than a CL suppression in the presence of LPS.

Example VII - Dose-Response of Whole Blood Chemiluminescence Reaction to Increasing Concentrations of Endotoxin at a Fixed Concentration of Antibody Utilizing Control (Non-Specific Murine Myeloma IgM) and Anti-LPS Murine IgM Antibody

Whole blood collected in EDTA vacutainer tubes was supplemented with LPS at concentrations of 0, 5, 50 and 500 pg/ml. One hundred microlitres of undiluted blood was mixed with 200 μl of luminol solution and 50 μl of antibody solution containing either non-specific control antibody or anti-endotoxin antibody at a concentration of 0.2 μg/ml. The reaction mixtures were pre-incubated with antibodies for 5 minutes at 37°C and then 50 μl of opsonized zymosan was added and the CL response monitored for a period of 60 minutes (Table 7a, b, c and d).

Example VII examines whether a differential CL dose-response could be achieved under assay conditions which would readily detect small concentrations of endotoxin. As indicated in Table 7, there was not a significant difference in the initial velocities of the CL response in the

absence of endotoxin, the mean slope for the linear portion of the control curves was 0.114 x 10^6 cpm/min, for the anti-LPS curves the mean slope was 0.133 x 10^6 cpm/min). As shown in Table 7, at a dose of LPS of 5 pg/ml of whole blood the mean slope for the anti-LPS antibody curves was 0.159 x 10^6 cpm/min, while the slope for the control antibodies remained at 0.114 x 10^6 cpm/min. In Table 7 the mean slope for the endotoxin containing curves was 0.219 x 10^6 cpm/min. at an LPS dose of 50 pg/ml of whole blood and 0.226 x 10^6 cpm/min at an LPS dose of 500 pg/ml of whole blood. The linear portion of the CL response curve for the control antibody curves remained constant at 0.116 to 0.113 x 10^6 cpm/min. These results demonstrate that a dose-response relationship can be generated for concentrations of LPS ranging from 5-500 pg/ml. Such a response could allow quantitative or semiquantitative detection of endotoxin in patient samples. With this assay protocol the utility of the CL assay was examined in ICU patients who had clinical evidence of sepsis or risk of sepsis and patients with no clinical evidence or at low risk of sepsis.

Example VIII - Initial Correlation Analysis Between Chemiluminescent Assay of Endotoxin and a Standard Reference Method Employing the Limulus Amebocyte Lysate (LAL) Assay

In this study arterial blood samples were taken from patients with clinical symptoms of sepsis into sterile EDTA containing vacutainer tubes and assayed for the presence of endotoxin by both the chemiluminescent whole blood assay and the reference limulus amebocyte lysate assay using the "Endospecy" assay kit purchased from BioWhittaker (Walkerville, MD, U.S.A.) or Seikagaku Kogyo Ltd. (Tokyo, Japan). Control samples were also obtained from non-septic patients and healthy ambulatory donors. Figure 3A uses blood from a patient with severe sepsis syndrome who died 6 hours after the sample was taken. Figure 3B uses blood from a healthy

ambulatory volunteer. Figure 3C uses blood from a patient with chronic sepsis. Figure 3D uses blood from a patient with severe sepsis syndrome who died 3 days after the sample was taken but had no evidence of gram-negative infection. Figure 3E uses blood from a patient with no clinical evidence of any septic foci. All blood transfer and reagent dispensing for both assays was accomplished using endotoxin free pipettes and sterile practice. The chemiluminescence assay mixture was composed of 200 μl of luminol solution, 50 μl of undiluted anti-coagulated whole blood, 50 μl of antibody (conc. 0.2 mg IgM/ml) and 50 μl of complement opsonized zymosan. All reagents were added in the order listed and the first three solutions were pre-incubated at 37°C for 5 minutes prior to the addition of zymosan, followed by the initiation of CL readings which were monitored for 60 minutes. All chemiluminescence assays were always run in conjunction with blood obtained from non-septic patients and ambulatory lab staff to verify the absence of false positive results. A positive control sample containing blood supplemented in vitro with E. coli LPS at a concentration of 100 pg/ml was always assayed with each run of patient samples.

Parallel blood samples from patients and controls were centrifuged at 700xg for 15 minutes to remove cells and duplicate 50 μl aliquots of plasma were removed using endotoxin free pipettes and transferred into endotoxin free glass test tubes for LAL assay. The plasma was treated with endotoxin free perchloric acid to remove inhibitory factors according to the procedure of Inada K., et al. CRC Review on Gram-negative Endotoxin 225 (1989) and subsequently assayed for endotoxin using the high sensitivity protocol as specified by Seikagaku Kogyo Inc (Toxicolor System Instruction Manual for Endotoxin Determination.

In Example VIII the response of the CL assay for endotoxin was compared to the standard Limulus Amebocyte Lysate (LAL) assay which is used by the pharmaceutical industry as a reference method for the detection

of gram-negative endotoxin. In this initial study we chose three patients who had clinical evidence of sepsis as determined by a certified Intensivist (a physician specializing in Intensive Care medicine) in the Surgical Intensive Care unit at The Toronto Hospital and two patients who had no clinical evidence of sepsis (one of these samples was obtained from a healthy ambulatory volunteer and the other sample from an ICU patient who was being weaned from respiratory support). Figure 3A displays the chemiluminescent response of blood taken from the radial artery of a patient with severe sepsis syndrome who died 6 hours after the sample was taken. The cause of death was hypotensive shock which was refractory to inotropic support. It is clear from the CL response in the presence of anti-LPS antibodies that this patient had a high level of endotoxin which was confirmed by LAL assay to be in the order of 753 pg/ml (see Table 8). Even with such a high levels of antigen which would result in high levels of mediators and thereby white blood cell activation, the antigen/antibody formation still cause an increase in white blood cell oxidant production. Figure 3B illustrates the CL profile of a healthy ambulatory volunteer and shows no differential response to anti-LPS antibody which was confirmed by LAL assay to indicate the absence of LPS in the blood. Figure 3C displays the CL response of a patient with chronic sepsis which was confirmed by blood culture to be primarily due to a beta haemolytic gram positive streptococcus. The CL assay indicated that this patient also had a response consistent with a low level of gram-negative septicemia which was below the limits of detection when assayed by LAL. The limit of detection using the LAL assay was a whole blood concentration of 50 pg/ml LPS. In order to remove interfering, substances the LAL assay requires a perchloric acid pre-treatment step which results in a ten fold dilution of the blood which is added to the assay mixture. This step poses a major limit on the analytical sensitivity of the assay. Figure 3D displays the CL response of a patient who had severe sepsis syndrome which ultimately contributed to his death 3

days after the blood sample used for the analysis was taken. The CL analysis indicated no evidence of LPS in the blood which was confirmed by LAL assay. The microbiological reports on culture material for this patient suggested that he had gram positive sepsis. Figure 3E represents the results of a CL assay for LPS conducted on blood obtained from a patient who was being weaned from respiratory support and was previously cachectic, but had no clinical evidence of any septic foci. The LAL assay confirmed the absence of endotoxin. These results suggest that the CL assay devised for the rapid detection of gram-negative endotoxin is capable of detecting LPS in patents with sepsis syndrome in whom LPS is detectable by standard LAL assay. In one patient (Figure 3C), gram-negative endotoxin was detectable by CL assay but probably below the limits of detection based on the LAL assay. The sensitivity and rapidity of the CL assay confirms its great potential in the early detection and clinical management of patients with sepsis syndrome.

In a further comparison of the present invention's CL method and the LAL assay, patients were tested for the presence of LPS at different times and using varying antibody dilutions. The LPS values for the CL assay for each test closely matched the values for the LAL assay. These LPS results for the CL assay and LAL assay are shown in Table 9.

Example IX - Chemiluminescent Response of Whole Blood from a Septic Patient Using Three Concentrations of Antibody

The patient had recurrent problems with a leaky duodenal ulcer. The patient experienced a temperature spike in the morning. The blood sample was taken approximately four hours before he was taken to the OR for abdominal cavity lavage.

A preferred approach for testing patient samples for endotoxin is based upon the following assay conditions: 20 microlitres of the patient's blood (EDTA anti-coagulated) is mixed with 20 μl (microlitre) of antibody

(three different dilutions are used, 0.2, 0.02 and 0.002 mg/ml in an endotoxin free assay cuvette. The mixture is incubated for 10 minutes at 37°C and then 200 μl of luminol solution (40 μM) is added (pre-equilibrated to a temperature of 37°C) followed by 50 μl of complement opsonized zymosan 2.5 - 3.0 x 10^9 particles/ml. Measurement of emitted light is then initiated in the chemiluminometer.

As demonstrated in Figure 4 (using the preferred patient assay format) a significant difference between control and anti-endotoxin antibodies can be achieved within 20 minutes. The assay is shown only for the antibody concentration of 0.2 mg/ml since the other antibody concentrations gave no differential response between control and anti-endotoxin antibody. The upper tracing in the Figure depicts the CL response of anti-endotoxin antibody containing blood, which the lower panel depicts the pattern achieved with a non-specific control antibody. The patient's sample was confirmed to contain 420 pg/ml of gram-negative endotoxin by LAL assay. The format of this assay was designed to minimize the amount of antibody necessary to evoke a significant chemiluminescence enhancement in the presence of gram-negative endotoxin. For this reason only patient sample and the antibody are incubated in the first phase of the reaction sequence in order to maximize effective antibody antigen complex formation. This preferred format has been adopted for patient studies.

Figure 4 demonstrates clearly the difference in the chemiluminescence levels for the patient as compared to the control using an antibody concentrations of 0.2 mg/ml.

Example X - Effects of a Maximum Stimulatory Concentration of C5a on Whole Blood Chemiluminescence in a Blood Sample Obtained from a Patient with Septic Shock and a High Blood Endotoxin Concentration (>700 pg/ml)

As indicated in Table 10, complement anaphylatoxin C5a at a dose which stimulates maximum opsonin receptor expression had no effect on the ratio of integrated light intensity observed in the presence of control antibody versus specific anti-LPS antibody. The concentration of anti-LPS IgM antibody and the irrelevant IgM antibody are 0.2 mg/ml and the concentration of C5a is 10 pg/sample. The patient chosen for this study had high levels of LPS (>700 pg/ml) in his blood as determined independently by LAL assay using the plasma assay procedure protocol of BioWhittaker Inc. (Walkerville MD) or Seikagaku Kogyo (Tokyo, Japan). A blood sample from this patient was obtained for assay of endotoxin using control and anti-LPS antibody in the presence and absence of complement opsonized zymosan. As shown in Table 10, the addition of C5a at a dose which recruits maximum opsonin receptor expression had no effect on the ratio of integrated light intensity observed with control versus anti-LPS antibody. The C5a was added to the blood samples immediately after the 5 minutes incubation of blood with antibody and the samples were then incubated an additional 5 minutes at 37°C prior to the addition of luminol solution. In the absence of opsonized zymosan, C5a increased the whole blood chemiluminescence of both control and anti-LPS assay samples but had no effect on the ratio of control versus anti-LPS antibody integrated light intensity. Similarly in the presence of complement opsonized zymosan, C5a had no effect on the same ratio. In addition C5a had a minimal effect on the opsonized zymosan containing assay tubes, causing only a slight increase in chemiluminescent light release. This is indicative of the fact that the patients blood already had virtually maximal opsonin receptor expression in the absence of C5a. The addition of C5a only increased the

chemiluminescent response by about 10% in assay samples containing opsonized zymosan. These results clearly demonstrate that the formation of antigen-antibody complexes in the form of LPS - anti-LPS antibody complexes in patient samples containing endogenous LPS and the subsequent effect of these complexes on white cell chemiluminescence is independent of opsonin receptor expression and proceeds via an activation pathway which does not depend upon differential opsonin receptor expression.

Example XI - Illustration of Optimal Antigen-Antibody Complementarity using a Clinically Significant Range of LPS Concentrations

These experiments summarized in Figure 5 demonstrate the dependence of the whole blood chemiluminescence response at antigen (LPS) concentrations of 0, 10, 100 and 1000 pg/ml upon the specific antibody concentration (Xomen E5 0.2, 0.02 and 0.002 mg/ml). The maximal chemiluminescence generated at an LPS conc. of 1000 pg/ml is achieved at an antibody concentration of 0.2 mg/ml (Figure 5a). The maximal response for an LPS concentration of 100 pg/ml is achieved at an antibody concentration of 0.02 mg/ml (Figure 5b) and for an LPS concentration of 10 pg/ml at an antibody concentration of 0.002 mg/ml (Figure 5c). As indicated in Figure 5, a baseline sample is included containing the specific antibody but no LPS. A baseline sample is included at each concentration of antibody used. This study was conducted using the standard assay format. Fifty microlitres of EDTA anti-coagulated whole blood was mixed with 50 microlitres of the respective antibody concentration and incubated at 37°C for 5 minutes followed by the addition of 200 microlitres of luminol solution and then 50 microlitres of complement opsonized zymosan. The integrated light intensity was collected for 30 minutes and is expressed on a relative scale in Figure 5. This experiment illustrates the dependence of the chemiluminescence signal on the optimal antigen - antibody complementarity.

Example XII - Effects of Heat Labile Factors in Plasma on the Chemiluminescence Response to Antigen - Antibody Complexes

In this experimental series the standard assay protocol was adopted using a monoclonal anti-LPS IgM antibody (Xomen E5) concentration of 0.2 mg/ml and an endotoxin concentration of 1000 pg/ml. The effect of heat treatment of the plasma prior to the formation of antigen - antibody complexes was examined. EDTA anticoagulated whole blood was centrifuged at 1500 x g for 15 min. to separate plasma from cells. The plasma fraction was then carefully removed and incubated for 30 minutes at 25°C or at 56°C and then added back to the cell fraction. Fifty microlitres of whole blood (Plasma fraction treated at room temperature or at 56°C) was then mixed with an equal volume of antibody solution and incubated at 37°C for 5 minutes with subsequent addition of luminol solution (200 microlitres) and complement opsonized zymosan (50 microlitres). The resulting chemiluminescence signal was then measured for 60 minutes and the peak CL count was recorded. An identical assay protocol using whole blood without centrifugation and heating was included for comparison. As shown in Table A, the prior heating of the plasma component of whole blood abolished the increased chemiluminescent response to antigen - antibody complexes. This experiment suggests that a heat labile component of plasma, likely a protein(s) of the complement pathway, is necessary for activation of increased white cell chemiluminescence following the formation of antigen - antibody complexes.

Example XIII - Lack of Chemiluminescent Response with E. coli in Whole Blood Unless Antibody is Added

E. Coli was isolated from a patient with gram negative sepsis. An isolate of the bacterium was cultured on agar and suspended in liquid medium at a density of approximately 1 million bacteria per ml. Aliquots of this mixture were added to whole blood and tested for chemiluminescence

response in the presence of murine ascites fluid containing an IgM directed against LPS epitopes. The source of the monoclonal IgM antibody was QED Laboratories in LaJolla, California, U.S.A. Experiments showed the ability to detect bacteria at concentrations of 10^4 bacteria per ml of whole blood and less using the IgM in murine ascites fluid added to whole blood. In these experiments the whole blood chemiluminescence response was augmented with opsonized zymosan. Samples containing bacteria and antibody together showed an augmented response compared to blood containing antibody alone. Bacteria added to blood had no effect on neutrophil chemiluminescence in the absence of antibody. E. coli in a concentration of 10^5/ml whole blood had no detectable effect on whole blood chemiluminescence after pre-incubation of whole blood for 30 or 60 minutes with the bacteria. The results are shown in Table 10.

Example XIV - Chemiluminescent Response Descreases with IgG for Hepatitis A

A murine monoclonal antibody to human Hepatitis A virus of the IgG class was tested for its ability to detect the virus in whole blood at antibody dilutions of 1/50, 1/200 and 1/1,000 against antigen dilutions of 1/50 and 1/500 of the stock viral antigen solution which contained approximately 100 μg/ml of viral protein. The Hepatitis A virus was killed and was at a protein concentration of 100 μg/ml and the anti-Hepatitis A antibody was a murine IgG at a protein concentration of 1 mg/ml. The Hepatitis A virus and antibody were available from ADI of Mississauga, Ontario, Canada. The test procedure was that of Example IX. In these experiments a converse suppressive effect of viral antigen-antibody complexes on whole blood chemiluminescence was observed. This effect may be due to the binding of antigen antibody complexes directly to the neutrophil Fc receptors followed by complement mediated cytolysis of the neutrophils by the membrane attack complex. Such a response would

diminish the chemiluminescence response in samples containing a combination of antigen and antibody, relative to antibody alone.

It is commonly believed that during bacterial infection phagocytes are activated by endotoxin and also cytokines. Experiments with antibodies against tumour necrosis factor (TNF) have detected this cytokine using the same principle employed in the endotoxin assay. This confirms the generic nature of the reaction sequence of this invention. This invention provides a diagnostic screening technology for detecting sepsis due to a variety of agents. The invention may be used with monoclonal antibodies against the following:

1) Bacteria

Gram-negative, for example with antibodies directed against Lipid A, O-saccharides or O-antigens,

Gram-positive, for example with antibodies directed against lipoteichoic acid,

2) Viruses and viral particles.

3) Fungae, in particular, Candida.

In addition, monoclonal antibodies against inflammatory mediators, such as, tumour necrosis factor, interleukins 1, 6, 8, Interferon γ and transforming growth factor β, will indicate the presence and degree of sepsis in this invention.

An added diagnostic feature of this invention is that it can be used to titrate a patient's therapeutic antibody titre and to evaluate the effectiveness of antibody therapy in reducing endotoxin levels. By conducting a simultaneous panel of tests using antibodies against TNF, IL-1, IL-6 and other markers of sepsis it will be possible to establish a "sepsis panel" to indicate the degree and magnitude of sepsis progression. A septic panel would use a combination of monoclonal antibodies against bacteria, virus or

fungae and inflammatory mediators. Each tube could contain a different monoclonal antibody to test the patient sample for a variety of septic indicators. One panel kit could include antibodies to the most common types of bacterial toxins, for example, antibody against gram-negative bacterial endotoxin and antibody against gram-positive bacteria lipotechoic acid. Another panel kit could have antibodies against the viral or fungal antigens which are associated with sepsis. Another panel kit could have antibodies against the inflammatory mediators to determine the patient's immune activation in response to sepsis.

Thus this invention allows for the diagnosis and staging of patients septic condition and also the evaluation of the efficacy of therapeutic treatments. A sepsis panel provides information on a range of sepsis indicators to better indicate the degree of progression of sepsis. Identifying those patients at risk of sepsis is important for initiating effective therapeutic treatment. The invention is easily performed, requiring only three reagents and a luminometer. This test can be used to serially monitor a patient, for example by conducting the test three or four times a day or more as clinical conditions or therapeutic interventions warrant.

TABLE 1

TURBIDOMETRIC ASSAY OF LPS-AntiLPS ANTIGEN-ANTIBODY COMPLEX FORMATION

(λ=340 nm. Absorbance at 5 min. time point x 10^{-2}

	LPS concentration (μg/ml)			
Antibody concentration (μg/ml)	100	10	1	0.1
200	6.31	8.48	7.96	8.1
20	2.8	2.6	2.58	2.53
2	0.33	0.14	0.1	0.11
0.2	0.08	0.01	0.02	0

TABLE 2

EFFECT OF VARYING AntiLPS ANTIBODY CONCENTRATIONS ON CHEMILUMINESCENT RESPONSE OF WHOLE BLOOD IN PRESENCE AND ABSENCE OF LPS

Values of chemiluminescence are given as 30 min. integral x 10^{7}

Antibody concentration (μg/ml)	**No LPS**	**LPS (1 ng/ml)**
100	3.07±0.20	2.03±0.22
10	3.09±0.13	2.85±0.06
1	4.04±0.02	4.22±0.07
0.1	4.64±0.14	4.65±0.15
0.01	4.90±0.19	5.71±0.36

TABLE 3

EFFECT OF LPS ON WHOLE BLOOD CHEMILUMINESCENT IN PRESENCE AND ABSENCE OF AntiLPS ANTIBODY

Values of chemiluminescence are given as 30 min. integral x 10^7

Panel A. No Antibody

NO LPS	LPS (1 ng/ml)
1.26±0.05	1.16±0.02

Panel B. Antibody (1.3 μg/ml)

NO LPS	LPS (1 ng/ml)
1.36±0.06	1.91±0.13

TABLE 4

EFFECT OF LPS ON WHOLE BLOOD CHEMILUMINESCENCE IN PRESENCE AND ABSENCE OF AntiLPS ANTIBODY

Values of chemiluminescence are given as 52 min. integral x 10^6

	LPS concentration (pg/ml)		
Antibody concentration (μg/ml)	0	100	1000
0.2000	1.47±0.01	3.11±0.03	1.05±0.06
0.067	1.80±0.13	2.56±0.13	1.93±0.10
0.022	2.35±0.20	2.27±0.05	3.51±0.32
0.007	3.25±0.22	2.02±0.09	5.58±0.46
0.002	3.34±0.13	1.95±0.11	3.07±0.21

TABLE 5

CHEMILUMINESCENT RESPONSE OF WHOLE BLOOD, CONTAINING *IN VITRO* ADDED LPS WITH ADDITION of AntiLPS ANTIBODY BUT NO ZYMOSAN

Values of chemiluminescence are given as 20 min. integral x 10^3

PANEL A. CL response without LPS

Control antibody	AntiLPS antibody (0.2 mg/ml)
9.76±0.90	36.4±1.52

PANEL B. CL response with added LPS (1 ng/ml)

Control antibody	AntiLPS antibody (0.2 mg/ml)
9.22±0.25	68.5±2.50

PANEL C. Effect of different LPS concentrations on whole blood chemiluminescence with AntiLPS antibody (0.2 mg/ml)

LPS concentration (pg/ml)	Chemiluminescence
0	48.8±1.40
10	61.7±3.20
50	62.9±2.70
100	100±2.20

TABLE 6

CHEMILUMINESCENT RESPONSE OF WHOLE BLOOD, TO *IN VITRO* ADDED LPS AT VARYING CONCENTRATION OF AntiLPS ANTIBODY

Values of chemiluminescence are given as 60 min. integral x 10^7

PANEL A. Preincubation of blood with LPS and AntiLPS antibody for 5 min.

Antibody concentration (μg/ml)	NO LPS	LPS (10 pg/ml)
0.002	4.39±0.71	4.66±0.16
0.02	3.09±0.64	3.88±0.69
0.2	3.86±0.45	5.40±0.37
2	4.19±0.62	4.55±0.59
20	3.84±0.54	3.71±0.41
200	3.98±0.30	7.68±0.56

PANEL B. Preincubation of blood with LPS and AntiLPS antibody for 1 hr.

Antibody concentration (μg/ml)	NO LPS	LPS (10 pg/ml)
0.002	4.81±0.40	5.20±0.30
0.02	5.48±0.21	5.07±0.46
0.2	5.29±0.46	5.02±0.58
2	5.15±0.14	5.06±0.07
20	5.35±0.42	5.26±0.61
200	6.82±1.46	3.45±0.18

TABLE 7

CHEMILUMINESCENT RESPONSE OF WHOLE BLOOD TO *IN VITRO* ADDED LPS AND AntiLPS (0.2 mg/ml)

Values of chemiluminescence are given as slope (c.p.m./min.x10^6) at 10 min.

PANEL A. No LPS added	
Control antibody	**AntiLPS antibody**
0.113	0.133
PANEL B. LPS (5 pg/ml)	
Control antibody	**AntiLPS antibody**
0.114	0.159
PANEL C. LPS (50 pg/ml)	
Control antibody	**AntiLPS antibody**
0.116	0.219
PANEL D. LPS (500 pg/ml)	
Control antibody	**AntiLPS antibody**
0.113	0.226

TABLE 8

LAL ASSAY FOR LPS:
RESULTS OF ANALYSIS ON ENDOTOXIN
STANDARDS IN WHOLE BLOOD AND PATIENT SAMPLES

Origin of Blood	Optical Density Mean Value	LPS Conc. pg/ml of Original Sample
blank	0.003	0
standard 1	0.166	1000
standard 2	0.087	500
standard 3	0.020	100
panel A	0.125	753
panel B	0.005	0
panel C	0.004	0
panel D	0.007	0
panel E	0.007	0

TABLE 9

COMPARISON OF LPS RESULTS BETWEEN CL METHOD AND LAL ASSAY IN PATIENTS WITH CLINICAL SEPSIS

Patient	CL Assay Result pg/ml LPS	Ab Dilution	LAL Assay pg/ml
M.O.	>100	1:10	130
M.O.	20-50	1:100	40
M.O.	>200	1:10	400
J.S.	20-50	1:100	50
J.S.	Neg.		Neg.
J.S.	>100	1:10	90
M.P.	>100	1:10	120
P.S.	Neg.		Neg.
P.S.	Neg.		Neg.
P.S.	Neg.		Neg.
J.V.	>200	1:10	>700
J.V.	>200	1:10	750
M.H.	20-50	1:100	60

TABLE 10

EFFECTS OF A MAXIMUM STIMULATORY CONCENTRATION OF C5a ON WHOLE BLOOD CHEMILUMINESCENCE IN A BLOOD SAMPLE CONTAINING A HIGH ENDOTOXIN CONCENTRATION OBTAINED FROM A SEPTIC PATIENT (LPS > 700 pg/ml)

Assay Components	Light Integral (30 min)	%CV	Ratio of Light Integral Control vs anti-LPS Ab
Patient Blood + Control Ab	1.2×10^5	16	
Patient Blood + anti-LPS Ab	2.3×10^5	15	0.52
Patient Blood + Control Ab + C5a	1.77×10^6	13	
Patient Blood + anti-LPS Ab + C5a	3.5×10^6	15	0.51
Patient Blood + Control Ab + opZym	5.45×10^8	7	
Patient Blood + anti-LPS Ab + opZym	14.9×10^8	4.9	0.37
Patient Blood + Control Ab + opZym + C5a	6.0×10^8	7.6	
Patient Blood + anti-LPS Ab + opZym + C5a	15.7×10^8	5.4	0.38

opZym = human complement opsonized zymosan

TABLE 11

EFFECTS OF HEAT TREATMENT OF PLASMA ON THE CHEMILUMINESCENCE SIGNAL MAXIMUM IN THE PRESENCE OF ANTIGEN - ANTIBODY COMPLEXES

Assay Components	Mean CL Signal Maximum (CPM)	%CV
Blood + anti-LPS IgM	2.4×10^6	3
Blood + anti-LPS IgM + Endotoxin	3.3×10^6 *	1.8
Blood + anti-LPS IgM (Plasma stored at 25°C)	2.1×10^6	2.5
Blood + anti-LPS IgM (Plasma heated at 56°C) + Endotoxin	2.1×10^6	2.3

* = $P < 0.01$ vs Blood + anti-LPS IgM, paired t test, n=6.

TABLE 12

E. COLI WHOLE BLOOD CHEMILUMINESCENCE EXPERIMENTS

Mass of Ab per reaction in mg	E. coli conc. Bacteria/ml blood	Light Integral	CV%	Peak CPM
0.014	10^5	$1.22x10^8$	8.7	$3.1x10^6$
0.014	None	$0.996x10^8$	15	$2.78x10^6$
0.0028	$5x10^4$	$1.30x10^8$	2.5	$3.55x10^6$
0.0028	None	$0.95x10^8$	16	$2.69x10^6$
0.0028	10^5	$1.4x10^8$	6.8	$3.7x10^6$
0.0028	None	$0.94x10^8$	16	$2.65x10^6$

TABLE 13

HEPATITIS A VIRUS CHEMILUMINESCENCE EXPERIMENTS

Antibody Dilution	Antigen Dilution	Light Integral	CV%	Peak CPM	CV%
1/50	1/50	$1.28X10^8$ *	1.2	$3.33x10^6$	1.7
1/50	No Ag	$1.46x10^8$ *	3.2	$3.60x10^6$	3.2
1/200	1/50	$1.31x10^8$ *	13	$3.98x10^6$	14
1/200	No Ag	$2.24x10^8$ *	8	$6.63x10^6$	8
1/1000	1/50	$1.33x10^8$ *	15	$3.87x10^6$	16
1/1000	No Ag	$2.81x10^8$ *	13	$8.52x10^6$	14
1/50	1/500	$1.62x10^8$ *	1.3	$5.0x10^6$	4
1/50	No Ag	$2.09x10^8$ *	7	$6.4x10^6$	5.3
1/200	1/500	$1.31x10^8$ *	4	$3.9x10^6$	4
1/200	No Ag	$1.5x10^8$ *	11	$4.6x10^6$	12
1/1000	1/500	$2.98x10^8$ *	2	$8.5x10^6$	2.8
1/1000	No Ag	$4.31x10^8$ *	5	$12.35x10^6$	4.7

*All differences were statistically significant by paired "t" test at $p<0.05$

Although preferred embodiments of the invention are described herein in detail, it will be understood by those skilled in the art that variations may be made thereto without departing from the spirit of the invention or the scope of the appended claims.

CLAIMS:

1. A method for determining the extent of an infection in a human or animal patient by detecting the amount of an antigen indicative of such infection, the amount of said antigen being detected in a patient blood derived test sample containing blood cell fractions, said method comprising:

i) incubating said test sample with an amount of test antibodies specific to said antigen indicative of infection to form antibody/antigen complexes which are insoluble in the sample,

ii) allowing said antibody/antigen complexes to interact with said white blood cell fractions which results in the production of oxidants,

iii) introducing to either steps i) or ii) a chemiluminescent compound to said test sample,

iv) said oxidants reacting with said chemiluminescent compounds to emit luminescent light from said test sample,

v) measuring said amount of emitted light over a predetermined period, and

vi) correlating extent of infection by comparison of the measured amount of emitted light of the test sample with measured amount of light emitted by a control sample which is treated the same as the test sample for steps i) to v) except that in step i) control antibodies are used which are of the same class as the test antibodies but are non-specific to said antigens indicative of infection.

2. A method of claim 1 wherein said sample is whole blood.

3. A method of claim 1 wherein said sample comprises white blood cell fractions derived from whole blood, said fractions including neutrophils, lymphocytes and/or monocytes.

4. A method of claim 1 wherein zymosan is added to said sample before step iv).

5. A method of claim 1 wherein opsonized zymosan is added to said sample before step iv).

6. A method of claim 1 wherein opsonized latex beads are added to said sample before step iv).

7. A method claim 1 wherein said chemiluminescent compound is selected from the group consisting of luminol, lucigen and pholasin.

8. A method of claim 1, wherein the test sample and control sample in step i) are incubated with three ratios of antibodies to solution, said ratios being 1:10, 1:100 and 1:1000, said comparison for measured light from test sample to measured light from control sample being correlated to antigen quantity with the ratio which provides greatest difference in light emissions.

9. A method of claim 8, wherein the antibody is a monoclonal antibody selected from the class of IgM and IgG.

10. A method of claim 9, wherein the antigen is derived from gram-negative bacteria, gram-positive bacteria, virus or fungus.

11. A method of claim 10, wherein the sample is whole blood.

12. A method of claim 11, wherein the viral antigen is Hepatitis A virus.

13. A method of claim 11, wherein the gram-negative bacterial antigen is endotoxin lipid A.

14. A method of claim 13, wherein the chemiluminescent compound is luminol.

15. A method of claim 14, wherein zymosan is added to enhance the reaction.

16. A method of claim 14, wherein opsonized zymosan is added to enhance the reaction.

17. A method of claim 14, wherein opsonized latex beads are added to enhance the reaction.

18. A diagnostic kit for use in determining the extent of an infection in a human or animal patient by detecting the amount of antigen indicative of an infection in a patient's blood derived test sample containing white blood cell fractions, said kit comprising:

i) a first container of antibody specific for antigen indicative of infection,

ii) a second container of chemiluminescent compound, and

iii) a third container of zymosan.

19. A diagnostic kit of claim 18 wherein said antibody is of the IgM class.

20. A diagnostic kit of claim 18 wherein said antibody is of the IgG class.

21. A diagnostic kit of claim 18 wherein said chemiluminescent compound is selected from the compounds consisting of luminol, lucigen and pholasin.

22. A diagnostic kit of claim 19, wherein said IgM antibody is against gram-negative endotoxin Lipid A.

23. A diagnostic kit of claim 20, wherein said IgG antibody is against Hepatitis A virus.

24. A diagnostic kit of claim 18 wherein said zymosan is opsonized.

25. A method for determining the extent of sepsis in a human or animal patient by detecting the amount of sepsis associated markers in a patient blood derived test sample containing white blood cell fractions, said method comprising:

i) incubating said test sample with an amount of test antibodies specific to a selected sepsis associated marker to form antibody/marker complexes which are insoluble in the sample,

ii) allowing said antibody/marker complexes to interact with said white blood cell fractions which results in the production of oxidants,

iii) introducing to either steps i) or ii) a chemiluminescent compound to said test sample,

iv) said oxidants reacting with said chemiluminescent compounds to emit luminescent light from said test sample,

v) measuring said amount of emitted light over a predetermined period, and

vi) correlating extent of infection by comparison of the measured amount of emitted light of the test sample with measured amount of light emitted by a control sample which is treated the same as the test sample for steps i) to v) except that in step i) control antibodies are used which are of the same class as the test antibodies but are non-specific to said sepsis associated markers.

26. A method of claim 25 wherein said sample is whole blood.

27. A method of claim 25, wherein said sample comprises white blood cell fractions derived from whole blood, said fractions including neutrophils, lymphocytes and/or monocytes.

28. A method of claim 25 wherein the test sample and control sample in step i) are incubated with three ratios of antibodies to solution, said ratios being 1:10, 1:100 and 1:1000, said comparison for measured light from test sample to measured light from control sample being correlated to antigen quantity with the ratio which provides greatest difference in light emissions.

29. A method of claim 28, wherein the antibody is a monoclonal antibody selected from the class of IgM and IgG.

30. A method of claim 29, wherein the sepsis associated marker is an inflammatory mediator.

31. A method of claim 30, wherein the inflammatory mediator is selected from the group consisting of tumour necrosis factor, interleukin-1, interleukin-6, interleukin-8, and interferon and transforming growth factor β.

32. A method of claim 31, wherein the sample is whole blood.

33. A diagnostic kit for use in determining extent of sepsis in a human or animal patient by detecting the amount of a sepsis associated marker in a patient's blood derived test sample containing white blood cell fractions, said kit comprising:

i) a first container of antibody specific for the sepsis associated marker,

ii) a second container of chemiluminescent compound, and

iii) a third container of zymosan.

34. A diagnostic kit of claim 33, wherein said sepsis associated marker is an inflammatory mediator.

35. A diagnostic kit of claim 34, wherein said inflammatory mediator is selected from the group consisting of tumour necrosis factor, interleukin-1, interleukin-6, interleukin-8 and interferon and transforming growth factor β.

36. A diagnostic kit of claim 34, wherein said antibody is of the IgM class.

37. A diagnostic kit of claim 34, wherein said antibody is of the IgG class.

38. A diagnostic kit of claim 34, wherein said chemiluminescent compound is selected from the compounds consisting of luminol, lucigen and pholasin.

39. A diagnostic kit of claim 34, wherein said zymosan is opsonized.

40. A method for detecting the amount of an antigen in a human or animal patient's body fluid test sample in the presence of white blood cells and all complement proteins, said method comprising:

i) incubating said test sample with an amount of test antibodies specific to said antigen to form antibody antigen complexes which are insoluble in the sample,

ii) allowing said antibody/antigen complexes to interact with said white blood cells and said complement which results in the production of oxidants,

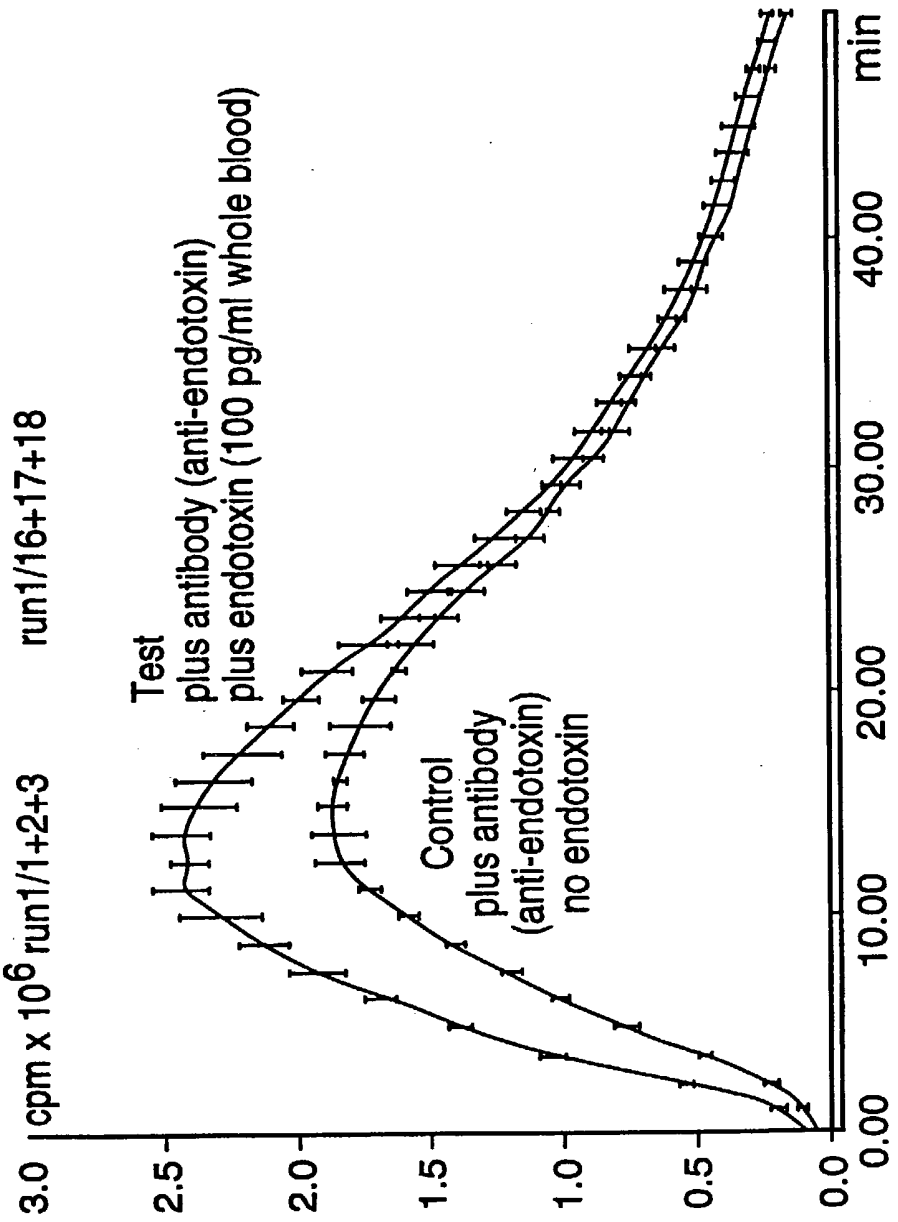
iii) introducing to either steps i) or ii) a chemiluminescent compound to said test sample,

iv) said oxidants reacting with said chemiluminescent compounds to emit luminescent light from said test sample,

v) measuring said amount of emitted light over a predetermined period, and

vi) correlating the amount of said antigen by comparison of the measured amount of emitted light of the test sample with measured amount of light emitted by a control sample which is treated the same as the test sample for steps i) to v) except that in step i) control antibodies are used which are of the same class as the test antibodies but are non-specific to said antigen.

41. A method of claim 52, wherein the white blood cells are neutrophils.


cpm x 10^6 run1/1+2+3
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20.00
30.00
40.00
min
Test
plus antibody (anti-endotoxin)
plus endotoxin (100 pg/ml whole blood)
Control
plus antibody
(anti-endotoxin)
no endotoxin

FIG.1.

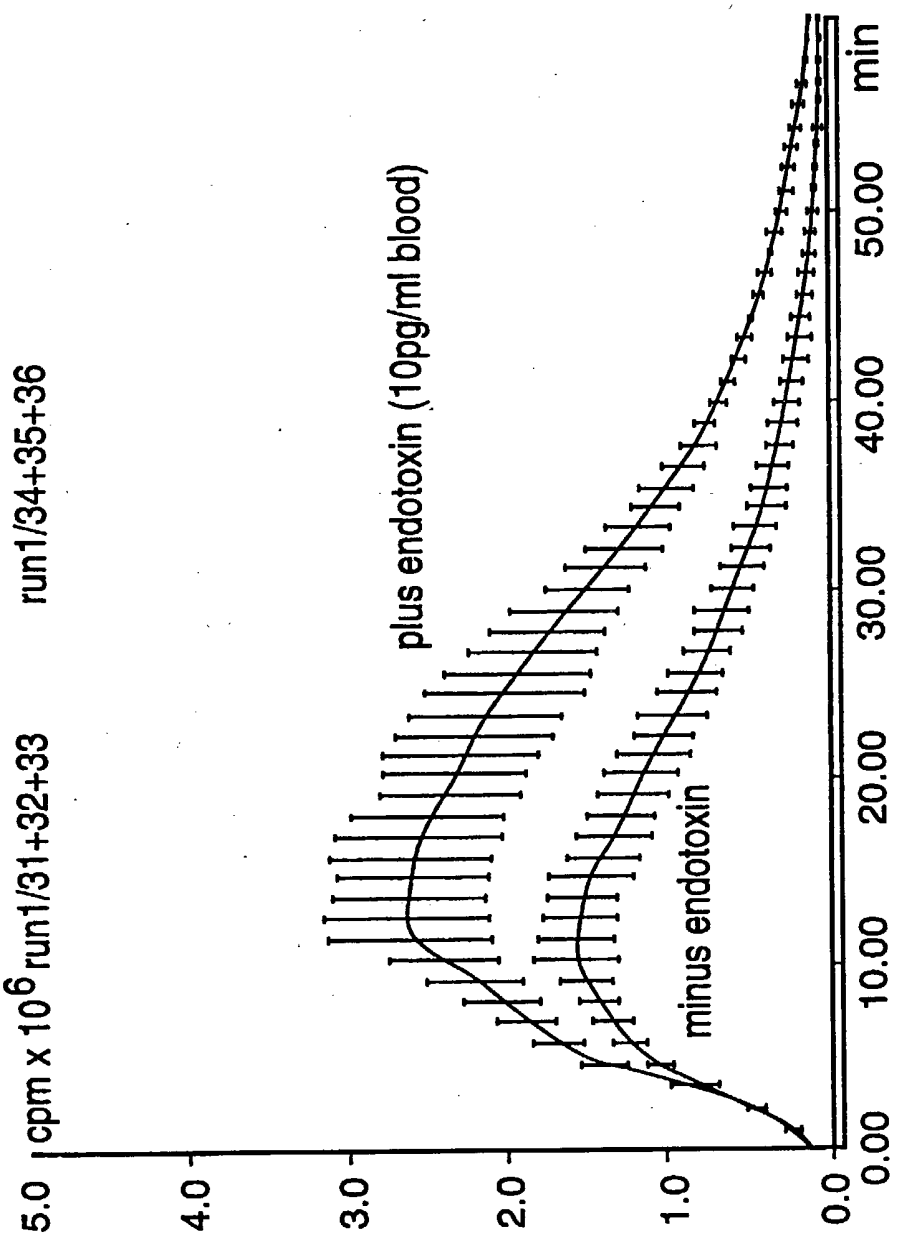
cpm x 10^6 run1/31+32+33
run1/34+35+36
plus endotoxin (10pg/ml blood)
minus endotoxin
5.0
4.0
3.0
2.0
1.0
0.0
0.00
10.00
20.00
30.00
40.00
50.00
min

FIG.2A.

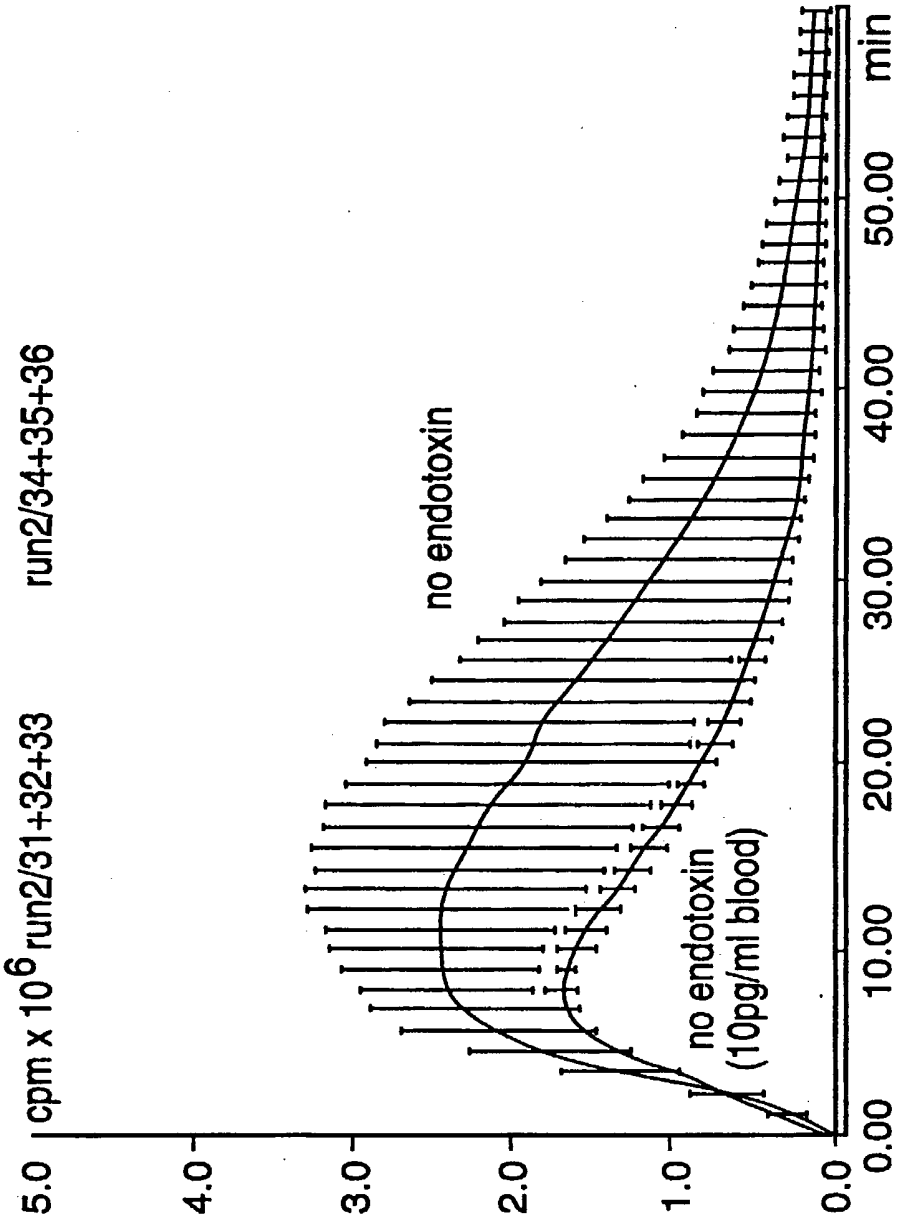
cpm x 10^6
run2/31+32+33
run2/34+35+36
no endotoxin
no endotoxin
(10pg/ml blood)
5.0
4.0
3.0
2.0
1.0
0.0
0.00
10.00
20.00
30.00
40.00
50.00
min

FIG.2B.

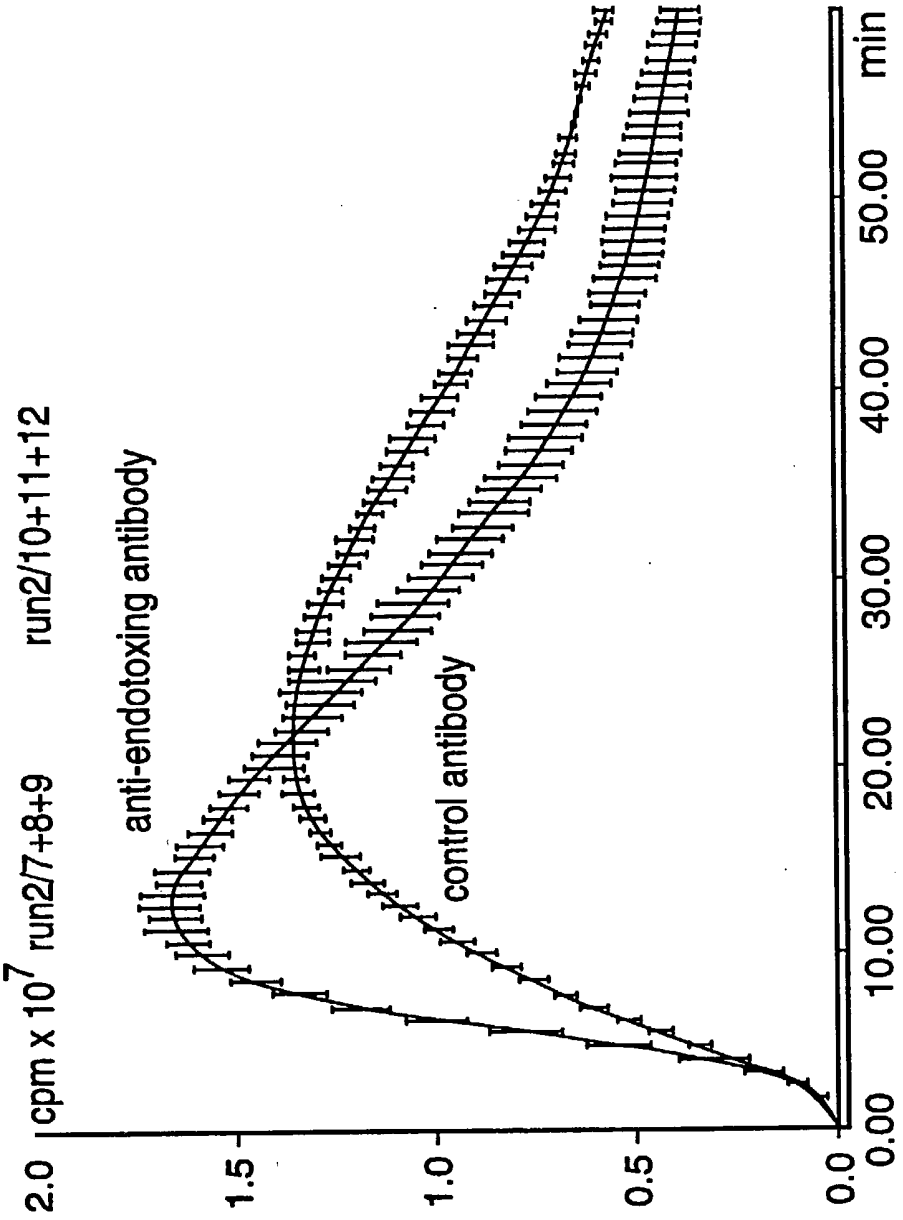
cpm x 10^7
run2/7+8+9
run2/10+11+12
anti-endotoxing antibody
control antibody
2.0
1.5
1.0
0.5
0.0
0.00
10.00
20.00
30.00
40.00
50.00
min

FIG.3A.

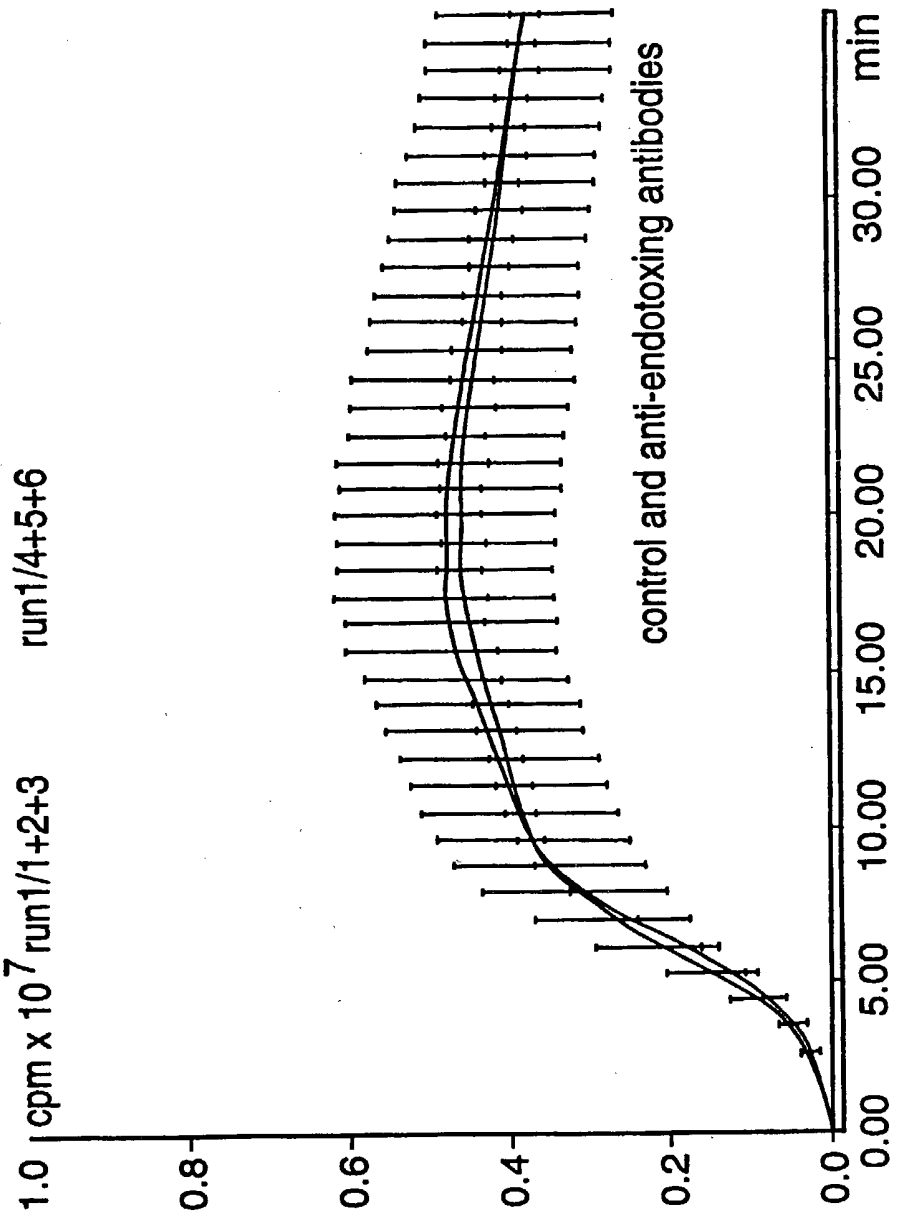
cpm x 10^7 run1/1+2+3
run1/4+5+6
control and anti-endotoxing antibodies
1.0
0.8
0.6
0.4
0.2
0.0
0.00
5.00
10.00
15.00
20.00
25.00
30.00
min

FIG.3B.

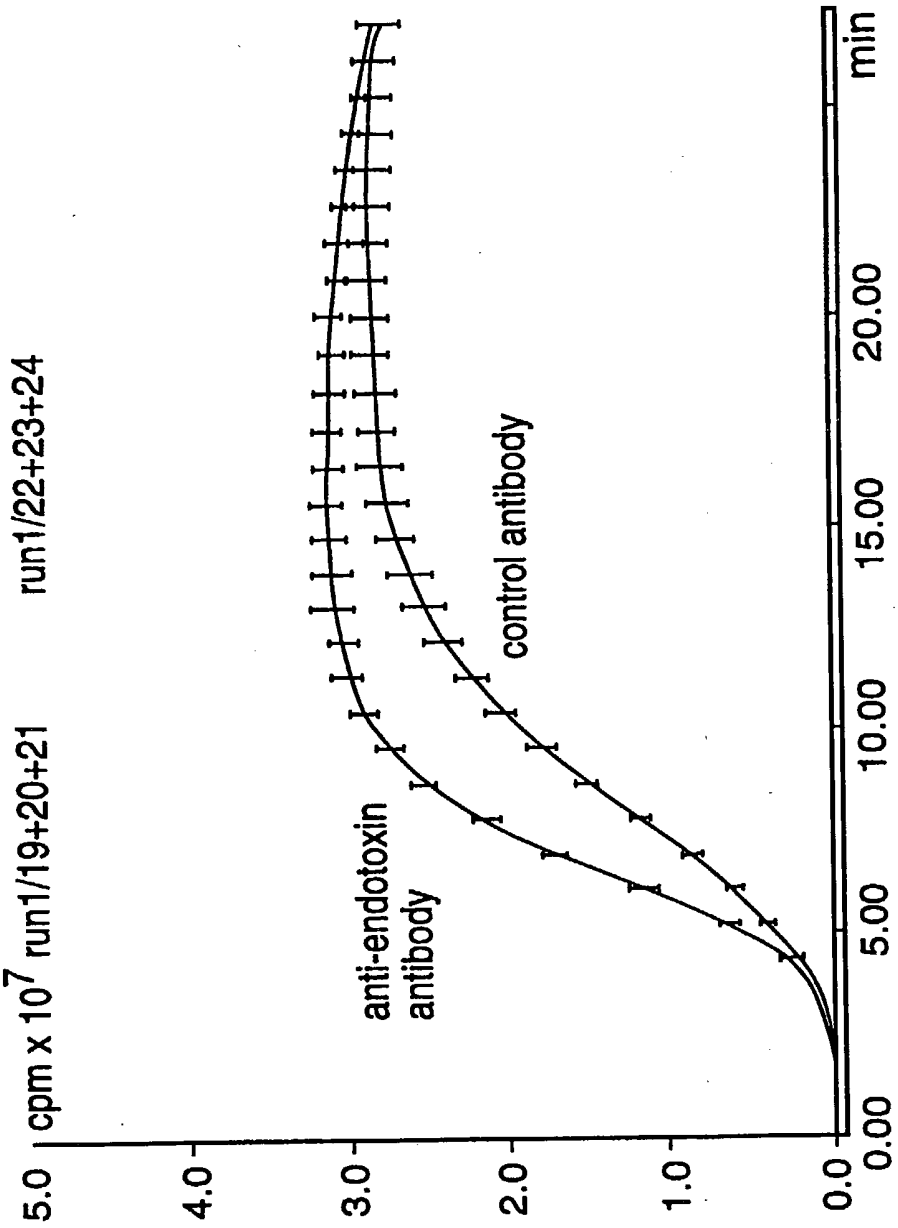
cpm x 10^7 run1/19+20+21
run1/22+23+24
5.0
4.0
3.0
2.0
1.0
0.0
0.00
5.00
10.00
15.00
20.00
min
anti-endotoxin antibody
control antibody

FIG.3C.

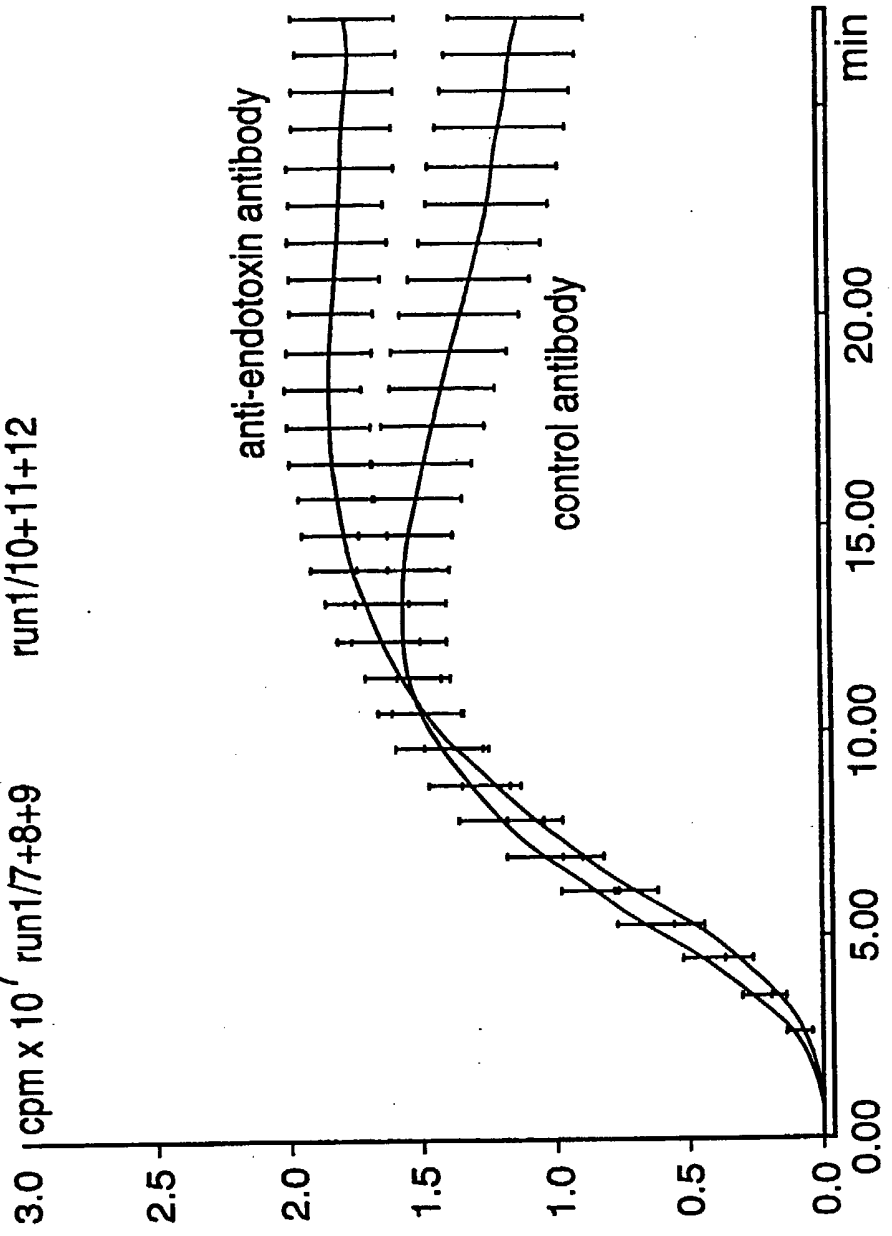
cpm x 10^7
run1/7+8+9
run1/10+11+12
anti-endotoxin antibody
control antibody
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0.00
5.00
10.00
15.00
20.00
min

FIG.3D.

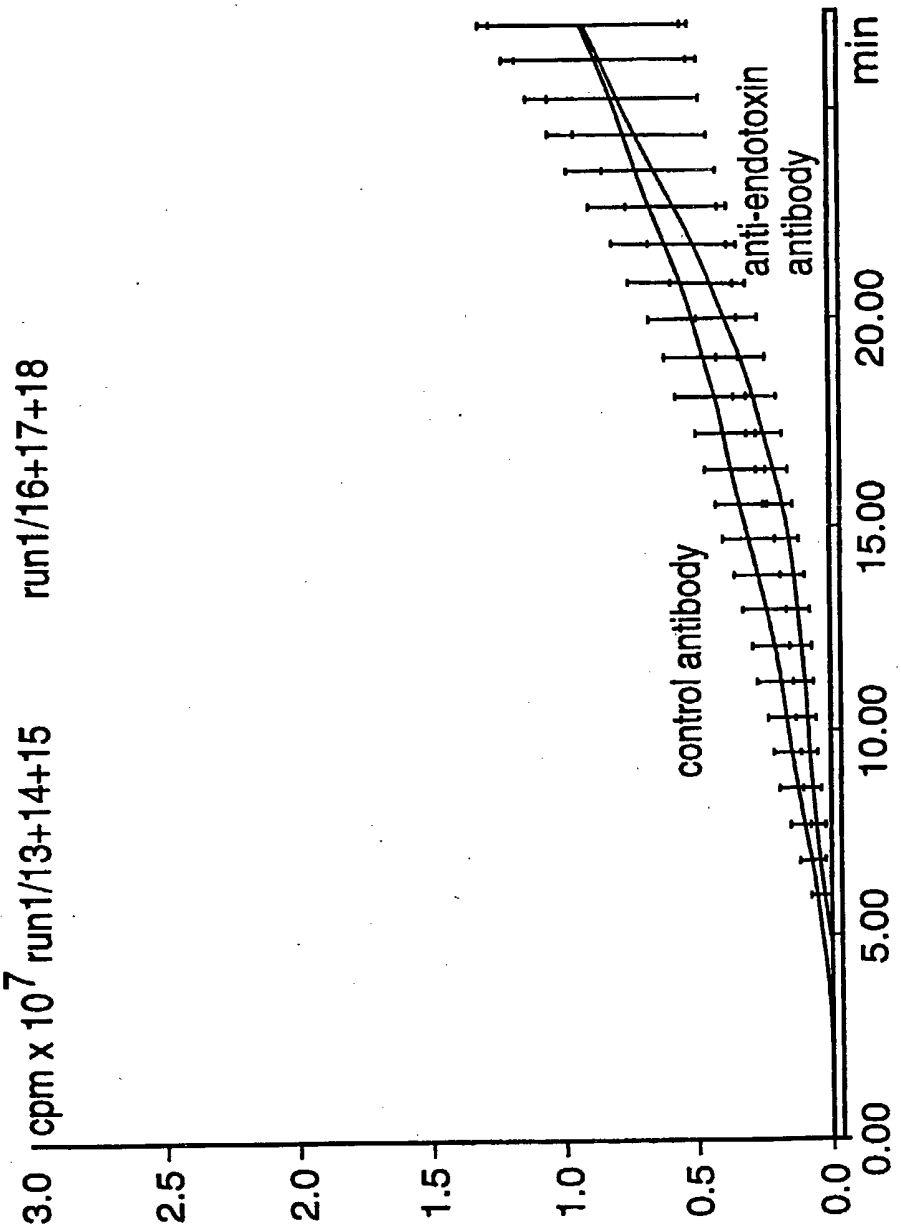
cpm x 10^7 run1/13+14+15
run1/16+17+18
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0.00
5.00
10.00
15.00
20.00
min
control antibody
anti-endotoxin antibody

FIG.3E.

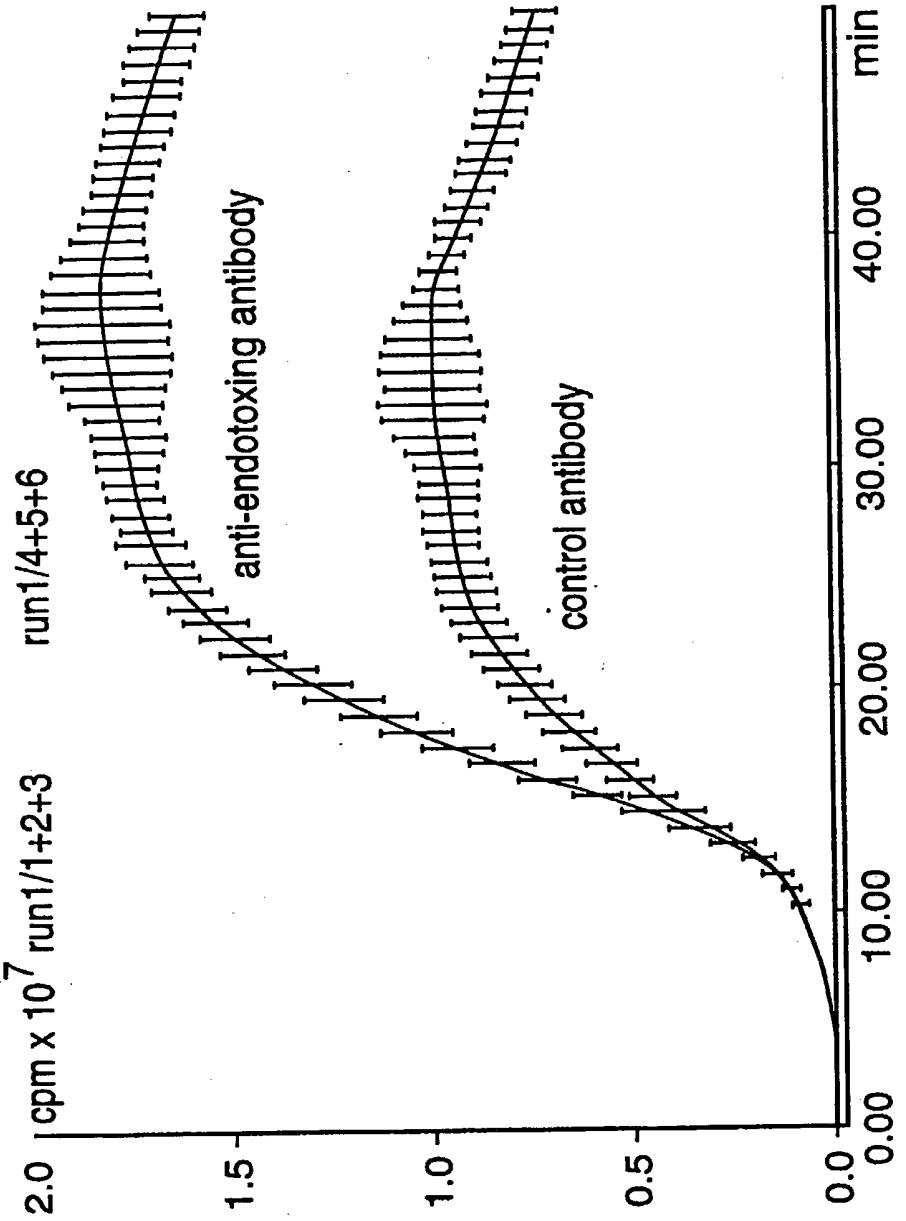
cpm x 10^7
run1/1+2+3
run1/4+5+6
anti-endotoxing antibody
control antibody
2.0
1.5
1.0
0.5
0.0
0.00
10.00
20.00
30.00
40.00
min

FIG.4.

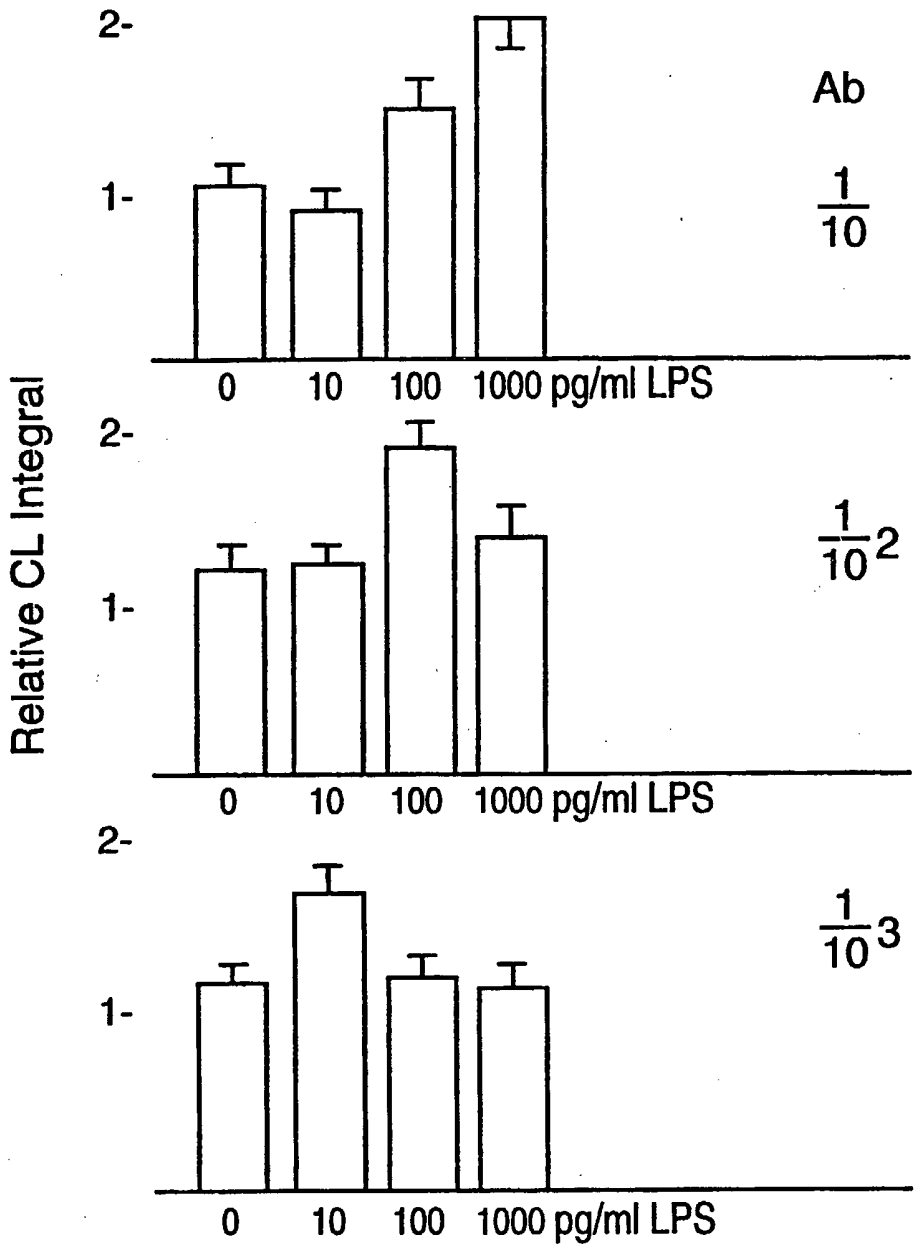
Relative CL Integral
2-
1-
Ab
1/10
0 10 100 1000 pg/ml LPS
2-
1-
1/10 2
0 10 100 1000 pg/ml LPS
2-
1-
1/10 3
0 10 100 1000 pg/ml LPS

FIG.5.

INTERNATIONAL SEARCH REPORT

Interr nal Application No
PCT/CA 94/00325

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 G01N33/569 G01N33/50 G01N33/52 G01N33/68 G01N33/577
G01N33/576

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP,A,0 430 440 (EXOXEMIS, INC.) 5 June 1991 see the whole document & US,A,5 108 899 (..) cited in the application	1-41
A	US,A,4 647 532 (H. WATANABE ET AL.) 3 March 1987 see the whole document	1-41
A	WO,A,92 03734 (A. DE WECK ET AL.) 5 March 1992 see the whole document	1-41
A	WO,A,90 06514 (CENTOCOR, INC.) 14 June 1990 cited in the application see abstract; claims	1-41

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 October 1994	28. 10. 94

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Griffith, G

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INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/CA 94/00325

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US,A,4 959 302 (A. J. CORNABY ET AL.) 25 September 1990 see the whole document ---	1-41
A	GB,A,2 131 948 (PACKARD INSTRUMENT COMPANY INCORPORATED) 27 June 1984 ---	
A	WO,A,92 16553 (NEW YORK UNIVERSITY ET AL.) 1 October 1992 cited in the application ---	
P,A	US,A,5 294 541 (D. S. KAPLAN ET AL.) 15 March 1994 -----	

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter nal Application No
PCT/CA 94/00325

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
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		AU-A-	6644390	31-05-91
		CA-A-	2028605	01-05-91
		JP-T-	5501452	18-03-93
		WO-A-	9106864	16-05-91
US-A-5108899	28-04-92	AU-A-	6644390	31-05-91
		CA-A-	2028605	01-05-91
		EP-A-	0430440	05-06-91
		JP-T-	5501452	18-03-93
		WO-A-	9106864	16-05-91
US-A-4647532	03-03-87	NONE		
WO-A-9203734	05-03-92	NONE		
WO-A-9006514	14-06-90	US-A-	5223395	29-06-93
		EP-A-	0446304	18-09-91
		JP-T-	4502212	16-04-92
US-A-4959302	25-09-90	NONE		
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		CA-A-	1210328	26-08-86
		DE-A-	3344607	14-06-84
		FR-A-	2537723	15-06-84
		JP-A-	59132361	30-07-84
		SE-A-	8306808	15-06-84
WO-A-9216553	01-10-92	AU-A-	1764992	21-10-92
		EP-A-	0610201	17-08-94
US-A-5294541	15-03-94	NONE		